Molecular basis of hepatic metaplasia of the pancreas

Shen, Chia-Ning

Award date:
2002

Awarding institution:
University of Bath

MOLECULAR BASIS OF HEPATIC METAPLASIA OF THE PANCREAS

Submitted by Chia-Ning Shen, MSc

for the degree of PhD

of the University of Bath

2002

Chia-Ning Shen

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ACKNOWLEDGEMENTS

Firstly, the deepest gratitude is to my supervisor Professor Jonathan Slack and Dr. David Tosh who proposed this interesting project and provided me with excellent supervision and support throughout. Secondly, thanks must go to Dr. Paul Martin and Dr. Andrew Ward for spending time on the viva and reading through my thesis.

During this project I have been very fortunate to receive a great deal of help and encouragement from the superb people in Jonathan's group. I would like to thank everybody who has helped me, including those who are still in the group and those that have now moved on.

Many thanks to Dr Itaru Kojima for the AR42J-B13 cells, Dr Calvin Swift for the elastase promoter plasmid, Dr Steve McKnight for the C/EBPβ plasmid, Dr Ueli Schibler for CMV-LIP and CMV-LAP plasmids and Professor Jonathan Seckl for pancreatic samples. Also thank to Dr Birgit Lane, Dr Ann Burchell and Dr Mike Coughtrie for antibodies. Special thanks to Overseas Reseach Students Awards Scheme and University of Bath for financial support.

Finally, I am grateful to my mother, my wife Fen-Ju and my sister Chia-Rui, for their limitless love, patience and support.

LIST OF ABBREVIATION

Albumin	Alb
α1-antitrypsin	α1-AT
Bromo-2-deoxyuridine	BrdU
CCAAT/Enhancer binding protein	C/EBP
Dehydroepaindrosterone sulphotransferase	DHEAST
Dexamethasone	DEX
α-fetoprotein	AFP
Glucose-6-phosphatase	G6Pase
Green Fluorescence protein	GFP
Hepatocyte growth factor	HGF
Hepatocyte nuclear factor	HNF
Pancreatic duodenal homeobox-1	PDX-1
Liver active protein	LAP
Liver-enriched transcription factors	LETFs
Liver inhibitory protein	LIP
Multi-drug resistant gene (also p-glycoprotein)	MDR
Oncostatin M	OSM
Phenol sulphotransferase	PST
Transferrin	TFN
Transthyretin (or prealbumin)	TTR

SUMMARY

Appearance of hepatic foci in the pancreas has been described in animal experiments and in human pathology. This work presents evidence to show that pancreatic cells can be converted into hepatocytes by glucocorticoid treatment. This occurs both in a pancreatic cell line, AR42J-B13, and in organ cultures of pancreatic buds from mouse embryos. The results demonstrate that metaplasia can be caused by a change to the environment of the cells and need not involve somatic mutation.

Using the AR42J-B13 cells we have established several features of the mechanism of the transdifferentiation: (1) a proportion of the hepatocytes arise directly from differentiated exocrine cells. This is shown by incorporating a GFP gene driven by the elastase promoter into the AR42J cells. Although the elastase promoter is switched off during the transformation some of the cells remain transiently green due to perdurance of the GFP. (2) Many of the hepatocytes are formed with no intervening cell division, shown by a failure to incorporate BrdU over the period of the transformation. (3) The molecular events associated with the transformation are induction of C/EBPβ followed by the nuclear translocation of HNF4α and the activation of differentiated hepatic products. Transfection of C/EBPβ into the cells can itself provoke the transdifferentiation while LIP (liver inhibitory protein), the dominant-negative form of C/EBPβ can inhibit the process, suggesting that C/EBPβ is a key component distinguishing between the liver and pancreatic programs of differentiation. (4) Epithelial morphogenesis plays an important role in hepatic transdifferentiation of pancreas: addition of cytochalasin D, an inhibitor of actin polymerisation, can prevent epithelial morphogenesis and liver gene expression.

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 Definition of metaplasia

Metaplasia is the name used to describe the conversion in postnatal life of one tissue type to another (Slack, 1986; Okada 1991). This term was introduced following anatomical and histological observation of the unexpected appearance of foreign tissues in ectopic sites. Therefore, "metaplasia" in some cases may be due to selective outgrowth of the minor cell types originally contained in a given organ. In other cases, metaplasia may be brought about by the differentiation of unspecific stem cells, or by a switch of pre-existing differentiated cells. Only the last categories should be counted as true metaplasia. In order to distinguish this case from overgrowth of one cell type by another, a term "cellular metaplasia" is often used.

Another term, transdifferentiation, was first introduced to denote a switch in cell differentiation by Selman and Kafatos (Selman et al., 1974). They observed a cell switch occurred normally in insect development. Okada and Eguchi identified the switch of pigment cells to lens as "transdifferentiation", which was convincingly demonstrated through *in vitro* clonal cell culture (Eguchi 1975; Araki and Okada, 1977). Based on their definition, transdifferentiation is an irreversible switch of one type of differentiated cell to another type of normal differentiated cell. There are two important criteria that need to be established: (1) The differentiated states before and after the transdifferentiation must be clearly defined which requires both morphological and molecular characterization; (2) The cell lineage relationship between the two cell types needs to be established.

Cellular metaplasia (or transdifferentiation) can be defined simply as a switch (or reprogramming) of cells, which have already expressed specific differentiated traits into another cell type distinguished from the original cells, by a set of phenotypic

characters. The wider class of metaplasias also includes cases in which stem cells of one tissue type switch to become those of another (Slack and Tosh, 2001).

1.2 Introduction to metaplasia – importance in development and disease

Metaplasia is always found in association with tissue damage and regeneration. It happens because there are errors leading to the cells changing their state of developmental commitment. Metaplasia in epithelia are common and do in fact often consist of a conversion of a patch of tissue into another type that arose as an adjacent rudiment in the embryo (Table 1.1, Slack 1985). For example, patches of ectopic intestinal epithelium are found in the stomach (Matsukura et al., 1980) or colonic type epithelium in the urinary bladder (Ward 1971).

During embryogenesis, tissues that develop as neighbouring rudiments in a common cell sheet will have similar combinations of transcription factors defining their commitment and may differ by the expression of just one gene. A previous theoretical work suggested that metaplasias might result from somatic mutation of homeotic genes normally required to distinguish tissue rudiments from one another (Slack 1985). Assuming that cells are indeed the same as the original embryonic progenitors for the tissue, then a change of state of such a gene in later life would cause the cells to "flip" from producing one tissue to producing another (Fig. 1.1; Slack 2000: Slack and Tosh, 2001).

Table 1.1 List of examples of epithelial metaplasia

(Table adapted from Slack, 1986)

Ectopic tissue	Location	Condition
Intestine	Stomach	Intestinal Metaplasia in a atrophic gastritis
Gastric (body type)	Duodenum and jejunum	Gastric metaplasia in duodenitis
Intestine (colonic)	Urinary bladder	Cystitis glandularis from Brunn's nests
Endocervical	Ovary	Mucinous cystadenoma
Tubal	Ovary	Serous cystadenoma
Endocervical	Vagina	Vaginal adenosis

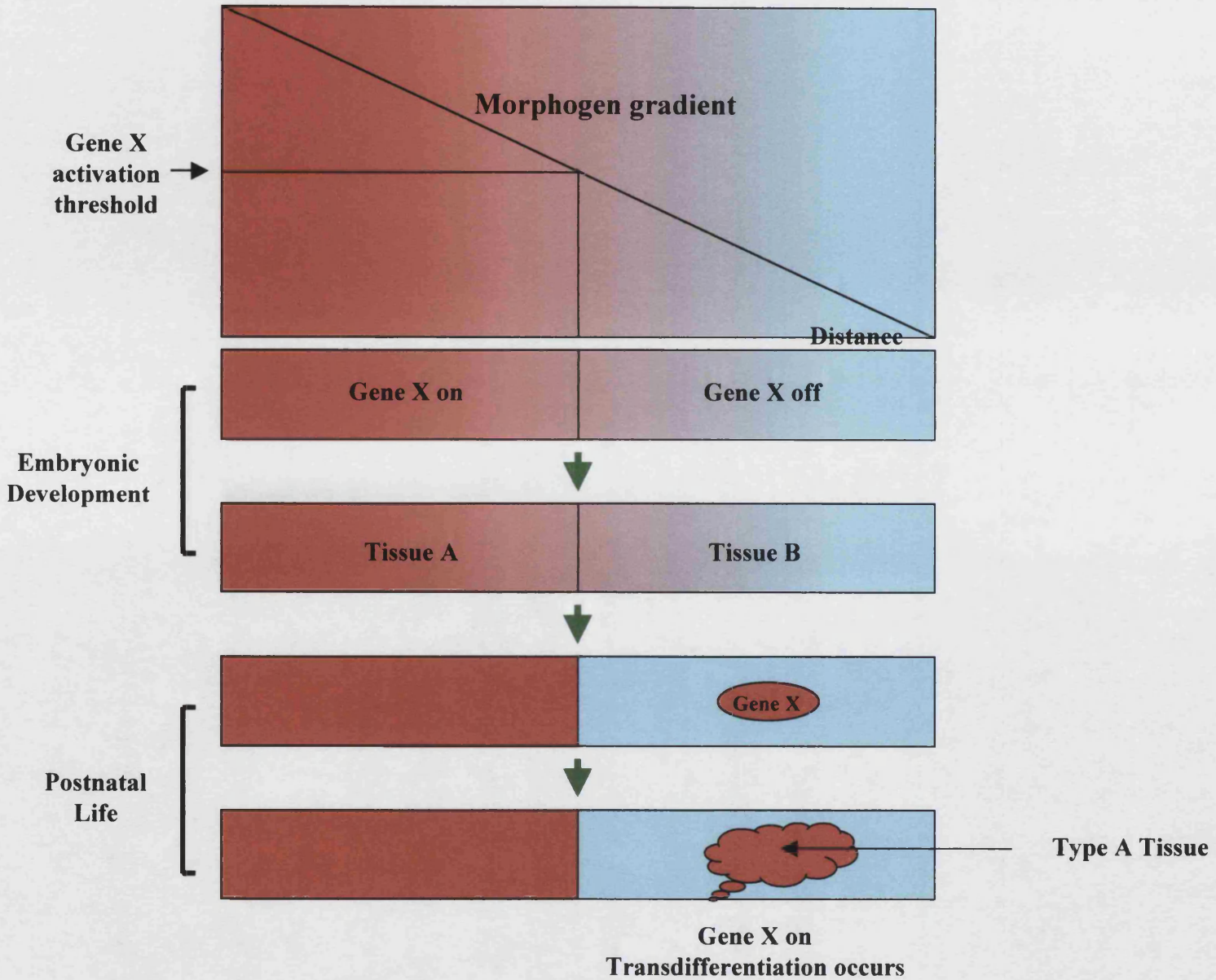

Morphogen gradient
Gene X activation threshold
Distance
Gene X on
Gene X off
Embryonic Development
Tissue A
Tissue B
Gene X
Postnatal Life
Type A Tissue
Gene X on
Transdifferentiation occurs

Fig 1.1 Mechanism of transdifferentiation in postnatal life. In the embryo, two tissue types arise from a common cell sheet because the master switch gene, X is activated in A tissue through induction by a morphogen but not in the other. In a postnatal life, either a change in the cellular environment or somatic mutation turns this gene X on in one or a few cells of the B tissue, then a metaplasia will result. (The figure was modified from Slack 2000)

For example, the homeobox gene *Cdx2*, a homologue of the Drosophila gene *caudal*, has been implicated in the control of cell differentiation in the intestinal epithelium. Recently, Furness and colleagues have shown that mice in which one allele of the Cdx2 gene had been inactivated by homologous recombination developed multiple intestinal polyp-like lesions in the colon (Beck et al., 1999). These lesions that didn't express Cdx2 were composed of heterotopic oesophageal-type epithelium together with some stomach and small intestinal mucosa. The result suggests Cdx2 directs endodermal differentiation toward a caudal phenotype. The haploinsufficient levels of expression in the developing distal intestine leads to metaplasia to a more rostral endodermal phenotype, such as forestomach epithelium that does not express Cdx2 during normal development

Since metaplasia represents a switch in developmental commitment of one tissue type to another, investigating metaplasia provides a novel opportunity to explore the cellular and molecular mechanisms underlying specification of different organs during the development.

1.3 Hepatic metaplasia of the pancreas

The appearance of hepatic foci in the pancreas occurs in rats, hamsters, mice, monkey and human in various conditions (Table 1.2). For example copper depletion of the diet (Rao et al., 1986c), or following transplantation (Dabeva et al., 1997), or in transgenic mice overexpressing Keratinocyte Growth Factor in the pancreas (Krakowski et al., 1999). It has also been observed naturally in the vervet monkey (Wolfe-Coote et al., 1996) and in human pancreatic tumours (Paner 2000). Although these whole organism observations cannot prove definitively that the hepatocytes

derive from pancreatic cells, they do suggest the likelihood of a relatively high frequency conversion between pancreatic cells and hepatocytes. Pancreatic-type tissue has also been found to occur in the livers of rats treated with polychlorinated biphenyls (Rao et al., 1986a), in fish liver tumors induced by chemical carcinogens, or in the liver of a human patient with hepatic cirrhosis (Wolf et al., 1990). The interconversion between pancreas and liver may reflect the close developmental relationship between the two tissues.

Liver and pancreas both originate from the foregut endoderm (Zaret, 1998; Slack, 1995). Although recently FGFs have been suggested to be implicated in the specification of liver development (Jung et al., 1999), the mechanism regulating endodermal epithelium to develop into pancreas and liver remains unclear. Presumably, there might be a gene(s) which distinguishes these two tissues in normal development. Under certain conditions, a discrete change in the expression of this gene might cause the hepatic metaplasia of pancreas in postnatal life. As a result, the system of hepatic metaplasia of pancreas provides a novel opportunity to elucidate the cellular and molecular mechanisms that control the development of two important endodermal organs-liver and pancreas.

Table 1.2 Examples of hepatic metaplasia in the pancreas

Species	Condition or Treatment	References
Hamster	Single dose of NBOP in regenerating pancreas.	Scarpelli and Rao1981
Rat	Fed with Ciprofibrate	Reddy et al., 1984
Rat	Copper-depletion-repletion.	Rao et al., 1986c; Rao et al., 1988
Monkey	Partial obstruction of main duct by cellophane wrapping.	Wolfe-Coote 1996
Mice	KGF (FGF-7) transgenic mice (driven by Insulin promoter)	Krakowski 1999
Human	Malignant islet tumour	Paner 2000
Human	Ductal carcinoma	Paner 2000

One of first animal models to describe that pancreatic cells can be converted into hepatocytes was performed by Rao and colleagues (Scarpelli and Rao, 1981). While they were trying to use a methionine-deficient protocol to induce regeneration of pancreas in Syrian golden hamster, they found that regenerating pancreatic cells were converted into hepatocyte-like cells, as judged by the presence of albumin, peroxisomes, and a variety of morphological features. These cells are stable after the conversion has been triggered by a single dose of the carcinogen *N*-nitrosobis (2-oxopropyl) amine administered during the S phase in regenerating pancreatic cells. This suggests that, given the proper stimulus, regenerating cells in adult pancreas can be redirected into a totally different pathway of differentiation to hepatocytes.

Treatment of rodents with peroxisome proliferators (e.g. Ciprofibrate) (Rao et al., 1982; Reddy et al., 1984), certain carcinogens (Rao et al., 1988b; Monis et al., 1991; Waalkes et al., 1992), or copper depletion followed by copper repletion (Rao et al., 1986c; Rao et al., 1988a; Dabeva et al., 1995) can generate hepatocytes in the pancreas. The most extensively studied *in vivo* model of the hepatic metaplasia of pancreas is the copper depletion-repletion protocol (Rao et al., 1988a). This is a highly reproducible *in vivo* model, in which hepatocytes were induced in the pancreas of adult rats that were maintained on copper-deficient diet containing a relatively non-toxic copper-chelating agent, Trien (triethylenetetramine tetrahydrochloride)(0.6% w/w) for 7-9 weeks and then returned to normal rat chow. Alternative Cu chelates (e.g. D-penicillamine) have been used, but those proved to be more toxic. The copper depletion results in almost complete loss of pancreatic acinar cells at the end of copper- depletion regimen, and in the development of multiple foci of hepatocytes during the recovery phase. In some animals, liver cells occupied more than 60% of

pancreatic volume within 6-8 weeks of recovery (Rao et al., 1988a).

It is believed that copper deficiency in rats leads to global acinar cell loss due to apoptosis. It possibly causes perturbations in cell-cell interactions and the microenvironment (Rao et al., 1993). Loss of acinar cells was associated with the proliferation of ductular epithelial and oval-like cells (similar to the oval cells in the liver- the progenitors of the liver stem cell). Massive depletion of the acinar cell pool creates severe expansion pressure on oval and ductular cells to fill the vacuity (Rao *et al.*, 1989). This probably causes a change in the commitment of these cells resulting in transdifferentiation into hepatocytes (Rao and Reddy, 1995).

1.4 Origin of pancreatic hepatocytes

The pancreas is an organ containing exocrine and endocrine tissues. The exocrine tissue is composed of the acinar cells secreting the digestive enzymes (e.g. amylase, lipase, and elastase) together with the epithelial cells of the pancreatic ducts which transport the digestive enzymes from the pancreas to the small intestine. These ducts may perhaps be the site of residence of pancreatic stem cells responsible for renewing the tissue (Ramiya et al., 2000). The endocrine cells are mostly organised into the islets of Langerhans and are subdivided into different cell types depending on the hormone that they secret (α cells: glucagon, β cells: insulin, δ cells: somatostatin, PP cells: pancreatic polypeptide). The question arises here if hepatocytes arise from differentiated pancreatic cells, then which type of pancreatic cells is the cell of origin? The whole animal experiments have suggested several different answers to this question.

Ciprofibrate, a peroxisome proliferator, can induce hepatocytes in the pancreas of adult male F-344 rats when added to their diet at a dosage of 10 mg/kg body weight for

60-72 weeks (Reddy et al., 1984). These cells are morphologically indistinguishable from hepatic hepatocytes and were usually localized adjacent to islets of Langerhans with extensions into surrounding acinar tissue. A significant increase in the volume density of peroxisomes, together with immunochemically detectable amounts of two peroxisome-associated enzymes, was observed in pancreas of rats maintained on ciprofibrate. Uricase-containing crystalloid nucleoids which are specific for rat hepatocyte peroxisomes were present. These structures facilitated the identification of cells with hybrid cytoplasmic features characteristic of both pancreatic acinar or endocrine cells and hepatocytes. These cells are presumed to represent a transitional state in which pancreas-specific genes are being repressed while liver-specific ones are simultaneously expressed. The presence of exocrine and/or endocrine secretory granules in such transitional cells indicates that acinar or endocrine cells represent the precursor cell from which pancreatic hepatocytes are derived.

Another suggestion is that pancreatic ductular epithelium in the hamster is the cell of origin (Makino et al., 1990). In the copper depletion-repletion rats, during the depletion period, there was an increase in the number of adipocytes in the interstitium, and in the number of interstitial and ductular cells. After 7 weeks of copper deficiency, albumin mRNA is localized over interstitial and ductular cells. These cells resemble morphologically the epithelial progenitors of the liver (Rao et al., 1989; Dabeva et al., 1995).

Finally, in a recent study, Krakowski generated transgenic mice in which KGF was driven by the insulin promoter (Krakowski et al., 1999). Remarkably, they found that ectopic KGF expression resulted in the emergence of hepatocytes within the islets of Langerhans in the pancreas. Additionally, significant intra-islet duct cell proliferation in the pancreas of transgenic KGF mice was observed. The unexpected

appearance of hepatocytes and proliferation of intra-islet duct cells in the pancreas of these mice might suggest endocrine cells have the potential to transdifferentiate into hepatocytes upon local exposure to KGF.

So these three studies have each suggested a different cell of origin for the pancreatic hepatocytes: acinar, endocrine or ductular. However, it is difficult from the whole-animal experiments to establish conclusively the progenitor cell type of the hepatocytes because no labelling of individual cell lineages could be carried out.

1.5 Molecular basis of hepatic metaplasia of pancreas

Fibroblast growth factors and several families of transcription factors including hepatocyte nuclear factors (HNF) 1, 3 and 4 and CCAAT/enhancer binding proteins (C/EBP) have been shown to be important components of the differentiation process that culminates in the fully functional liver (Darlington 1999). It is not clear whether these factors are involved in the generation of hepatic metaplasia. So far, only Dabeva and Rao have tried to determine the mechanism using northern blotting. The results show the pancreatic ductular cells express HNF-1, HNF-3α, HNF-3β, HNF-4, C/EBPα, C/EBPβ, and C/EBPδ (Dabeva et al., 1995; Rio et al., 1996) which suggest these liver-enriched transcription factors (LETFs) may be involved in the mechanism underlying the process of metaplasia. However some of these transcription factors were also found to be present in the pancreas and other tissues (Cereghini, 1996). For example, the mRNA of HNF-4 and HNF-1 increased after 5 weeks copper depletion in those pancreatic epithelial progenitors, but HNF-4 and HNF-1 are also found to be expressed in the islet of Langerhans (Miquerol et al., 1994), especially HNF-1 is essential for insulin gene transcription (Emens et al., 1992; Wang et al., 1998).

If these transcription factors also regulate pancreatic phenotype, then the results do not provide us with a clear molecular mechanism to understand how these factors induce the conversion of pancreatic cells to hepatocytes. In order to understand whether the process of metaplasia is governed by the combined action of the LETFs, we need a model system to further analyse the process. Thus it might be very helpful if we can establish an *in vitro* model for the hepatic metaplasia of the pancreas.

1.6 Objectives of the current work

Numerous examples have been described that indicate hepatic metaplasia of pancreas is a reality and can occur in *vivo*, however very little is known of the molecular and cellular mechanisms involved. There are four questions that arise. Firstly, we would like to ask whether or not hepatic metaplasia is a true example of transdifferentiation? If so, it needs to fulfill the two important experimental criteria defined by Eguchi and Kodama- (1) The two differentiated states before and after the transdifferentiation must be clearly defined, which will require both morphological and molecular characterisation. (2) The cell lineage relationship between the two cell types needs to be established, usually using a tissue culture system. Secondly, are cell type transformations the result of somatic mutations or of the environmental reprogramming of cells? Thirdly, if this is not the case of metaplasia, do all such changes represent transformations between stem cells? Fourthly, what are the genes whose altered expression underlies the changes?

Using a suitable in vitro model will help to address these four questions. In the present study, I have firstly established an *in vitro* model, and used the model to establish morphological and molecular characterisation of the transdifferentiation process. Secondly, I have investigated the cell linage relationship to determine the

origin of the pancreatic hepatocytes. Thirdly, I have attempted to define each phase of the pathway leading to the conversion and determine whether somatic mutations or environment caused the transformation. Fourthly, I have determined the expression patterns of the gene(s) involved in hepatic metaplasia in developing pancreas.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell lines and Culture Conditions

AR42J is a rat pancreatic exocrine cell line purchased from ECACC (European Collection of Cell Cultures, Proton Down, U.K.). AR42J-B13 cell (kindly provide by Dr. I. Kojima, Tokyo, Japan) is a subclone of the parent line AR42J. Both cell lines were maintained in Dulbecco's modified Eagle's medium (Sigma, Poole, U.K.) containing 2 mM L-glutamine, 0.5 u/ml penicillin, 500ng/ml streptomycin, and 10% fetal bovine serum (Life Technologies, Glasgow, U.K.). The medium was renewal every 2- 3 days, and subculture was performed every 4-6 days at a ratio of 1:7-1:10. Cells were frozen in fetal bovine serum containing 10% DMSO, and kept in liquid nitrogen or at -80°C.

RIN-5F cell line, a subclone of the RIN-m rat islet cell line, was purchased from ECACC (European collection of cell cultures). Cells were maintained in RPMI 1640 medium (Sigma, Poole, U.K) containing 2 mM L-glutamine, 0.5 u/ml penicillin, 500ng/ml streptomycin, and 10% fetal bovine serum. The medium was renewed every 2-3 days. Subculture was performed every 6-8 days at a ratio of 1:3-1:5. Cells were frozen and stored in fetal bovine serum containing 10% DMSO, and kept in liquid nitrogen or at -80°C.

Dexamethasone (Dex) was added at 1nM-1mM as a solution in ethanol and medium changed every 2-3 days (control cells received ethanol alone). RU486 (BIOMOL, Plymouth, U.S.A) was added at 2.5 μM concentrations, the treatment commencing 1 hour before addition of the Dexamethasone. In some experiments, Hepatocyte growth factor (R&D system, Oxon, U.K.) or Oncostatin M (R&D system, Oxon, U.K.) was added as a solution in phosphate-buffered saline contained 0.1% bovine serum albumin at a final concentration of 100 pM or 10 ng/ml together with or without 1 μM Dex.

For the stability determination- AR42J-B13 cells were treated for 2 weeks with 1 μM Dex, then split and cultured with 1 μM Dex and 10 ng/ml OSM for a forther 4 days. Cells were then cultured in control medium without Dex, fixed after 4 days and 10 days. Secondly, AR42J-B13 cells were treated for 4 weeks with Dex, large cells (>2 fold) were then sorted by cell sorter. After cells were recovered, some clones were picked and then cultured with 1 μM Dex for a further 4-6 weeks. After splitting, cells were cultured with 1 μM Dex for a further 4 days, then Dex was removed from medium and cells were fixed after 14 days.

Cell growth was analysed by seeding $1x10^5$ cells in 35 mm dishes with or without Dex. Cells were trypsinised and subsequently counted in a haemocytometer.

2.2 Embryonic culture of mouse pancreatic buds

Cultures were set up as described in Percival and Slack (1999) with minor modification. Briefly the dorsal buds were isolated from E11.5 mouse embryos. To set up cultures, the coverslips were placed in 35 mm petri plates containing BME medium with Earle's salts, 20% fetal bovine serum and 50 μg/ml gentamycin (all were purchased from Life Technologies, Glasgow, U.K.). A stainless steel cloning ring of 3 mm internal diameter was placed over the fibronectin-coated area, and the pancreatic bud was dropped into the center. To ensure spreading during culture, the buds were turned if necessary with a fine needle so that the cut surface lay in contact with fibronectin. Cultures were maintained for up to 7 days at 37°C in 5% CO_2, with a change of medium everyday. The stainless steel ring was removed at the second day of culture.

Treatment with Dexamethasone was started after 48hrs in culture. The medium was changed each day with addition of Dex. All trans-retinoic acid (Sigma, Poole,

U.K.) was reconstituted in DMSO at 10mM and used at 0.1, 1 or 10 μM with or without 1 μM Dexamethasone. The medium was change daily with or with retinoic acid.

2.3 Immunofluorescence Analysis

For immunofluorescent staining, cells were cultured on noncoated glass coverslips, rinsed with PBS, fixed with 4% paraformaldehyde (PFA) in PBS for 30 min or ice cold Acetone/Methanol (1:1) solution for 5 min. For PFA fixed cells, cells were then permeabilised with 0.1% (vol/vol) Triton X-100 in PBS for 30 min, and incubated in 2% blocking buffer (Roche, East Sussex, U.K.) which contained 0.1% Triton X-100, then incubated sequentially with primary antibodies for overnight at 4°C and secondary antibodies for 3 hrs at room temperature. After incubation of secondary antibodies, cells were washed 3 times with PBS buffer, and then mounted in Gelvatol medium (prepared by dissolving 20g of polyvinyl alcohol, in 80mls of 10mM Tris [pH 8.6], and 3g of n-propyl gallate in 50mls glycerol followed by mixing and centrifugation at 7000 *g* to remove any undissolved particles). Alternatively, for staining of pancreatic bud cultures, the pancreatic buds were fixed in MEMFA (10% formaldehyde, 0.1M Mops, pH 7.4, EGTA 2mM, $MgSO_4$ 1mM) for 30-45 min at room temperature. They were then washed in PBS and stored in PBS at 4°C for up to a few days. Prior to immunostaining, the cultures were treated with 1% TX100 in PBS and then blocked as above. The details and working dilution of antibodies has been listed in Table 2.1 and Table 2.2

Specimens were observed either using a Zeiss confocal microscope (LSM510) or with a Nikon fluorescent microscope. Image collection from the Nikon was made

with a Hamamatsu C4880 cooled CCD camera and the images processed with Image Pro Plus and Photoshop.

G6Pase or albumin positive cells were counted on the basis of immunocytochemistry. Images were collected under the same conditions from at least five different regions of Dex treated cells by using the Zeiss LSM510 confocal microscope. The number of positive cells were counted manually, and the total numbers for each specimen are larger than 500. Values are the means±SD for 3 experiments.

For staining of mitochondria, cells were loaded with 1 μM Rhodamine 123 (Molecular probe, Leiden, Netherlands) for 10 min at room temperature, and then fluorescent signals were observed using a Zeiss confocal microscope (LSM510).

For staining of polymeric forms of actin, phalloidin-FITC (5 μg/ml) or phalloidin-TRITC (10 μg/ml) (both were purchased from Sigma, and dissolved in DMSO) were added to specimens 1 hr before mounting in the slides.

Table 2.1 Working dilution of primary antibodies

Antibody	Species	Supplier	Dilution
α_1-antitrypsin	Rabbit Polyclonal	Sigma	1:400
α-Fetoprotein	Rabbit Polyclonal	Biomeda	1:300
Albumin	Rabbit Polyclonal	Sigma	1:500
Albumin	Goat Polyclonal	Sigma	1:300
Amylase	Rabbit Polyclonal	Sigma	1:300
BrdU	Mouse Monoclonal	Sigma	1:200
E-cadherin	Mouse Monoclonal	Transduction	1:200
β-catenin	Mouse Monoclonal	Transduction	1:400
C/EBPβ	Mouse Monoclonal	Santa Cruz	1:150
C/EBPβ	Rabbit Polyclonal	Santa Cruz	1:500
Pan-cytokeratin	Mouse Monoclonal	Sigma	1:400
CK8	Mouse Monoclonal	Birgit Lane	undiluted
G6Pase	Sheep Polyclonal	Ann Burchell	1:500
GSK3β	Mouse Monoclonal	BD	1:200
Glucagon	Mouse Monoclonal	Sigma	1:100
GR	Rabbit Polyclonal	Santa Cruz	1:100
GFP	Mouse Monoclonal	Clontech	1:300
HNF-4α	Goat Polyclonal	Santa Cruz	1:150
Insulin	Guinea-pig Polyclonal	Sigma	1:300
IPF-1 (PDX-1)	Rabbit Polyclonal	Jonathan Slack	1:100
MDR	Goat Polyclonal	Santa Cruz	1:100
ST (DHEA)	Rabbit Polyclonal	Mike Coughtrie	1:500
ST (Phenol)	Rabbit Polyclonal	Mike Coughtrie	1:1000
Transferrin	Rabbit Polyclonal	Dako	1:200
TTR (Prealbumin)	Rabbit Polyclonal	Dako	1:100

Table 2.2 Working dilution of secondary antibodies

Antibody	Species	Supplier	Dilution
Anti-goat IgG FITC	Rabbit Polyclonal	Sigma	1:300
Anti-goat/sheep IgG FITC	Mouse Monoclonal	Sigma	1: 50 / 1:150
Anti-guinea-pig IgG TRITC	Rabbit Polyclonal	Sigma	1:500
Anti-rabbit IgG biotinylated	Swine Polyclonal	Dako	1:300
Anti-rabbit IgG TRITC	Swine Polyclonal	Dako	1:200
Anti-rabbit IgG FITC	Goat Polyclonal	Jackson	1:300
Anti-mouse IgG FITC	Horse Polyclonal	Vector	1:150
Anti-mouse IgG AMCA	Horse Polyclonal	Vector	1:100
Anti-mouse IgG Texas Red	Horse Polyclonal	Vector	1:150
Anti-mouse IgG biotinylated	Horse Polyclonal	Vector	1:300
Anti-goat IgG biotinylated	Horse Polyclonal	Vector	1:300
Anti-Sheep IgG Texas Red	Rabbit Polyclonal	Vector	1:150
Anti-Rabbite IgG FITC	Goat Polyclonal	Vector	1:200

2.4 BrdU incorporation

Bromo-2-deoxyuridine (BrdU) was purchased from (Sigma, Poole, U.K.). BrdU was added as a solution in water at a concentration of 10 μM. Dex was added overnight before BrdU was added, and medium containing fresh BrdU and Dex was changed thereafter every 2 days. Cells were fixed with 4% paraformaldehyde in PBS for 30 min, and then treated with 2N HCl for 20 min at 37°C in a water bath. Immunostaining was performed as described above.

2.5 Transfection and production of stable transfectants

The elastase promoter was a gift from Dr. Calvin Swift (Southwestern Medical School, Texas), The transthyretin promoter was provided by Dr. R. Costa (University of Chicago, Illinois). Both pcDNA3-Elas-GFP2 and pcDNA3-TTR-GFP2 were constructed by removing the CMV promoter from the pcDNA3-GFP2 and replaced with either the elastase promoter or transthyretin promoter. (Constructs were made by Dr. D. Tosh).

Stable transfectants of AR42J-B13 were generated with pcDNA3-Elas-GFP2 by using neomycin resistance. 1 x 10^6 cells were seeded in 35 mm dishes and grown at 37°C, 5% CO_2 for overnight, then transfected with 1.5 μg of DNA/dish using FuGENE 6 transfection reagent (Roche, East Sussex, U.K.) according to the manufacturer's instruction. Stable transfectants were selected in medium containing 800 μg/ml G418 (Life Technologies, Glasgow, U.K.). After 4 weeks, clones were individually picked, then expanded and maintained in medium containing 200 μg/ml G418.

Transient transfection was also carried out using FuGENE 6 Transfection Reagent with 2 μg of DNA per dish and incubated at 37°C 5% CO_2 for either 72 hrs or 120 hrs, Cells were then fixed and immunostaining was performed as described above.

For transfection studies using LIP to prevent transdifferentiation, cells were transfected with LIP and then treated for 4 days with 1 μM Dex and 10 ng/ml OSM. Alternatively, AR42J-B13 cells were treated with 1 μM Dex for 8 weeks. Cells were then trypsinised, resuspend in PBS, and loaded into cell sorter (Becton Dickinson). Normal cell size value (SSC) around 400, and the large cells (SSC> 800) were collected. Cells were pelleted down, and cultured in medium with 1 μM Dex for two weeks. These cells were exposed to 1 μM Dex and 10 ng/ml OSM for 5 days prior to transfection with LIP. Following transfection the cells were treated with 1 μM Dex and 10 ng/ml OSM for 72hrs.

For transfection studies using TTR-GFP to determine the stability of the transdifferentiation, AR42J-B13 cells were treated for 2 weeks with 1 μM Dex, then split and cultured with 1 μM Dex and 10 ng/ml OSM for 4 days prior to transfection with CMV-nucGFP or TTR-nucGFP followed by exposure to 1 μM Dex and 10 ng/ml OSM for a further 72 hrs. Cells were then cultured in control medium without Dex, and the dish was photographed each other day with the confocal microscope.

2.6 RT-PCR

Total RNA was extracted using the TRI reagent (Sigma, Poole, U.K.). Briefly, cells were seeded on 100mm petri dishes without or with 1 μM Dex treatment for 2d, 4d, 7d 14d. Cells were lysed directly on the culture dish by adding 1 ml of the TRI reagent. After addition of the reagent, the cell lysate was passed several times through a pipette to form a homogenous lysate. After homogenization, the homogenate was

centrifuged at 12,000 x *g* for 10 minutes at 4°C to remove the insoluble material. The clear supernatant was transferred to a fresh tube, and then 0.2 ml of chloroform was added to the tube. Covering the sample, it was shaken vigorously for 15 seconds and allowed to stand for 2-15 minutes at room temperature. It was then centrifuged at 12,000 x *g* for 15 minutes at 4°C. The aqueous phase was transferred to a fresh tube; 0.5 ml of isopropanol was added and mixed. The sample was allowed to stand for 5-10 minutes at room temperature, and then centrifuged at 12,000 g for 10 minutes at 4°C. The supernatant was removed and the RNA pellet was washed with 75% ethanol. The RNA pellet was dried for 5-10 minutes, dissolved in water, and then stored at -80°C.

Before carrying out the cDNA synthesis, the RNA samples were digested with RQ-1 DNase (Promega, Southampton, UK) to remove any contaminating genomic DNA. First strand complementary DNA was synthesized using MMLV reverse transcriptase (Life Technologies, Glasgow, U.K.). The primers used are listed in Table 2.3. The reactions were processed in a DNA thermal cycler in the presence of 1.5 μC of [α-^{32}P] dATP under the following conditions: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The number o f cycles was 27, except for β-actin (22 cycles). The products were separated on a 5% polyacrylamide gel, which was dried and exposed to X-ray film. In order to confirm the absence of genomic DNA, primers for β-actin span one small intron, so if contaminated genomic DNA, and then we will find a 477 bp fragment will be detected.

TABLE 2.3 Sequences of RT-PCR primers

Gene	Sense primer	Antisense primer	Size (bp)	Accession
β-Actin	TCCGTAAAGACCTCTATGCC	AAAGCCATGCCAAATGTCTC	353	J00691
p48	GCTCCTGGAGCATTTCCCG	CTGAGGAACTCTACCTCCGC	285	X98446
HNF-1	CGCATACAAGCCTGCTTCC	GAGACGACGGCTCATGAAG	148	L09648
HNF-3α	CAGACTCTGGACCACAGTG	TTCAGGTGCAGCTGAGATTC	163	X55955
HNF-3β	GTCAGGAGCACAAGCGAGG	TGTGATGATGTTGCTGCTCG	241	L09647
HNF-3γ	CAAAGAAAGGAAACAGTGCC	TTGGAAGGTGTTGATGTCTG	307	L09648
HNF-4	CTCAACTCATCCAACAGCC	AAGCACTTCTTGAGCCTGC	221	D10554
HNF-6	GATCAATACCAAAGAGGTGG	TTGGACGTCTGTGAAGACC	316 (α)	X96553
			394 (β)	Y14933

2.7 Histology and Immunhistochemistry

The pancreas of glucocorticoid treated rats was kindly provided by Prof. J. R. Seckl (Dept. of Medical sciences, Western general hospital.), the procedure was performed as described previously (Nyirenda et al, 1998). Briefly, mothers were treated throughout the 3rd week of gestation (from day 15 to day 21-22 (depending on when pups were born)). The dose of 100 μg/kg (body weight) dexamethasone or vehicle was administered subcutaneously each day, and then the rats were sacrificed 3 weeks postnatally. The pancreas was dissected free, fixed overnight in 4% formaldehyde, and embedded in paraffin wax.

Representative sections (7 μm) were stained with haematoxylin and eosin (H&E). In addition, serial sections were prepared and immunohistochemically stained for insulin or glucagon. Briefly, sections were rehydrated and endogenous peroxidase activity was blocked by incubating with 3% H_2O_2 for 30 min. Sections were then blocked for 1hr in 2% Roche blocking buffer prior to application of antibodies and incubated for 12-14h at 4°C with mouse monoclonal antibody to glucagon (1:300 dilution) or guinea-pig anti-bovine insulin (1:200 dilution). Biotinylated anti-mouse or anti-guinea-pig IgG (Vector Laboratories, Burlingame, U.S.A.) were diluted 1:300 in PBS and applied for 1 hr. The slides were then washed in PBS and incubated for 1hr with an ABC kit (Dako, Cambridge, U.K.). Immunoreactivity was localised by incubation with DAB (10mg in 15ml PBS), 0.01% H_2O_2 for up to 5 min and the reaction stopped by washing in PBS. Tissue sections were counterstained with haematoxylin.

For immunofluorescence staining on tissue sections, sections were permeabilised with 0.1% Triton X-100 in PBS for 1 hr and then blocked for at least 90 min in Roche blocking buffer. Each primary antibody was applied overnight at 4°C

and washed the following day three times with PBS, and the secondary antibody was applied for 3 hr at room temperature and the slides washed and mounted in Gelvatol medium. The details and dilution were decribed in Table 2.1, 2.2

Sections were examined under the Leica DMRB microscope. Images of stained specimens were collected with a spot camera drive (Diagnostic Instrument Inc.) and stored as 24-bit TIFF images. Pictures of fluorescent specimens were taken by using a Zeiss confocal microscope (LSM510).

CHAPTER 3

IN VITRO CONVERSION OF PANCREATIC CELLS INTO HEPATOCYTES

3.1 Introduction

The conversion of pancreatic cells to hepatocytes is a well-studied example of transdifferentiation in mammals. Metaplasia represents a switch in developmental commitment of one tissue type to another. Therefore, investigating the metaplasia of pancreas to liver provides a novel opportunity to explore the cellular and molecular mechanisms underlying the development of these two endodermal organs. Since Rao and colleagues first described that regenerating pancreatic cells in hamsters can differentiate into hepatocytes (Scarpelli and Rao, 1981). The process of metaplasia of pancreas to liver has been investigated for nearly 20 years, but the mechanism which is responsible for metaplasia remains unclear. The main reason is that, until recently, no reliable *in vitro* model existed for the hepatic metaplasia of pancreas.

It is believed that one tissue may be replaced by another in two ways: either by a switch of developmental commitment or by colonization with cells of different origin (Slack, 1986). Only the former is a true metaplasia. Different labs have been using various protocols to induce the appearance of hepatocytes in the pancreas (see Chapter 1; Scarpelli and Rao, 1981; Rao et al., 1982; Reddy et al., 1984; Rao et al., 1988b; Monis et al., 1991; Waalkes et al., 1992; Rao et al., 1986c; Rao et al., 1988a; Dabeva et al., 1995; Dabeva et al., 1997; Krakowski et al., 1999). If the hepatic metaplasia of pancreas is a real example of metaplasia, then two questions arise? First, do all such changes represent transformations between stem cells, or can changes also occur between actual differentiated cells (i.e. a true transdifferentiation)? Secondly, if hepatocytes arise from differentiated pancreatic cells, then which type of the cell is the origin of pancreatic hepatocytes? Unfortunately, it is difficult from whole-animal experiments to establish conclusively either the progenitor cell type of the hepatocytes or the molecular basis of the switch.

Eguchi and Kodama defined two important experimental criteria that need to be established for a process to be defined as transdifferentiation (Eguchi and Kodama 1993). First, the differentiated states before and after the transdifferentiation must be clearly defined, which will require both morphological and molecular characterisation. Second, the cell lineage relationship between the two cell types needs to be established, usually using a tissue culture system. Our approach to proving or demonstrating that pancreatic hepatocytes directly arise from pancreatic cells is to establish an *in vitro* model by using differentiated cell lines. If it proves possible to induce metaplastic changes in culture by altering the extracellular environment or by gene transfer, then it will be very useful for determining the mechanisms involved. The results obtained from animal experiments suggest that hepatocytes might arise either from exocrine, endocrine or duct cells (Reddy et al., 1984; Krakowski et al., 1999; Makino et al., 1990). Therefore, we here tried to use AR42J (rat pancreatic exocrine cells), Rin-5F (rat pancreatic β cells), and Capan-1 or PANC-1 (human pancreatic duct cells) to determine the potential of these cell types to undergo metaplasia.

3.2 Hepatic metaplasia in pancreatic AR42J-B13 cells

AR42J cells are derived from azaserine-induced malignant nodules from the rat pancreas (Longnecker et al., 1979). These cells possess both exocrine and neuroendcrine properties. Because of the short lifetime of dispersed nonimmortalised pancreatic acinar cells, this acinar cell line has been and still is a widely used culture system (Christophe 1994; Caplin et al., 1999; Harris et al., 1999; Dethloff et al., 2000; Thommesen et al; 2000).

AR42J cells secrete amylase and other digestive enzymes (Christophe, 1994). Interestingly, the cells also express neuroendocrine markers such as synaptophysin, and neurofilament protein. It can take up and release γ-aminobutyric acid (GABA) and posses an excitable membrane. When treated with activin A, a member of the TGF-β supergene family, AR42J cells extend neurites and express pancreatic polypeptide (Yasuda et al., 1994; Mashima et al., 1996a). When the activin A treatment is combined with betacellulin or hepatocyte growth factor (HGF), AR42J cells differentiate into β cells as determined by expression of insulin and glucose transporter 2 (GLUT-2) (Mashima et al., 1996a; Mashima et al., 1996b). AR42J-B13, a subclone of AR42J cells, are almost entirely converted to insulin-producing cells after the incubation with activin A and HGF (Mashima et al., 1996b).

Dexamethasone, a synthetic glucocorticoid, is known to enhance the differentiation of AR42J cells toward the exocrine phenotype, increases the number of zymogen granules, the level of amylase mRNA, and the secretion of amylase (Logsdon et al., 1985). In addition, dexamethasone also shows the ability to induce the synthesis of albumin, transferrin, tyrosine aminotransferase, and glucose-6-phosphatase in liver cells (Chou et al., 1988; Schmoll et al., 1996; Kamiya et al., 1999). In the present study, I initially investigated the effect of dexamethasone (Dex) on AR42J-B13 cells to see if Dex causes a similar effect on exocrine expression as the parent cell line-AR42J. To our surprise, the results show that the dexamethasone can convert AR42J-B13 cells into hepatocyte-like cells. Incubating the cells with 10nM Dex for 5 days increased amylase expression in a proportion of cells. However, we observed that some cells became larger and flatter. The expression of amylase was also reduced in these cells compared to some of their neighbours after 5-days treatment. After 2 weeks, they were extremely flattened and the expression of amylase was almost gone (Fig 3.1A-D).

After a 3 week treatment, they showed strong expression of albumin, a gene known to be expressed in liver cells (Fig 3.1E-H). Vehicle controls (ethanol alone) were always negative.

AR42J cells treated with dexamethasone grow more slowly than control cells (Logsdon et al., 1985). To determine whether the transdifferentiation is paralleled by growth arrest, we cultured the cells with different concentrations of Dex (1nM-1μM). (Fig.3.2A). In addition to studying the cell growth, we also stained for albumin (Fig.3.2B-F). Concentrations greater than 10nM caused a significant reduction in the growth rate from about 6 doublings/week to about 3 doublings/week and this was accompanied by changes in morphology (flattened and large), and expression of albumin (Fig 3.2A-F).

We found that all effects of Dex were blocked by concurrent treatment of the cells with 2.5 μM RU486, the glucocorticoid receptor antagonist (Fig 3.2G). This result indicates that the effects are exerted through the glucocorticoid receptor. We also tested cortisol, the natural form of glucocorticoid, which showed that it had similar effects to Dex (results not shown).

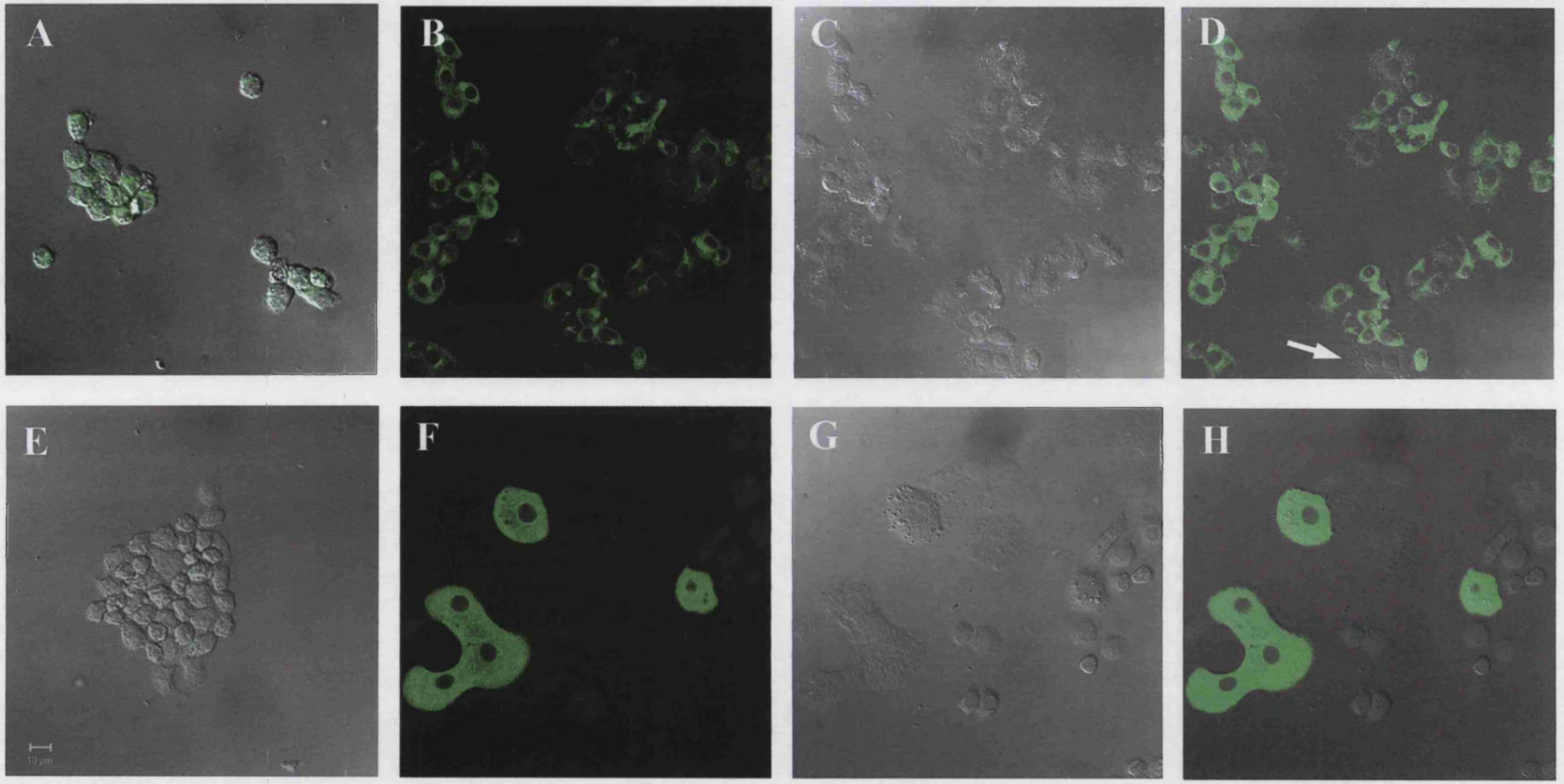
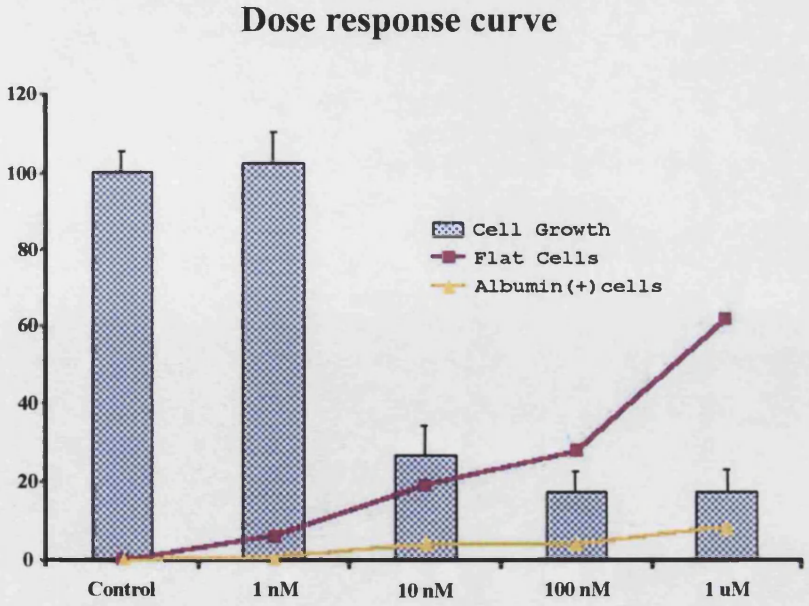
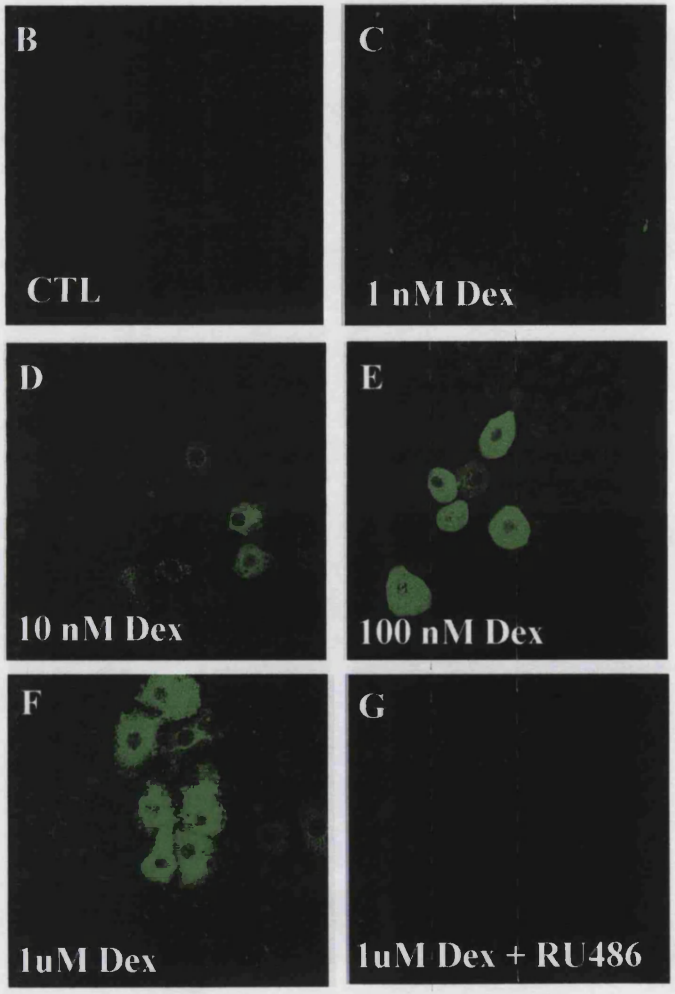

A
B
C
D
E
F
G
H
10 µm

Fig 3.1 Dexamethasone treatment leads to formation of a flattened, epithelial-like, morphology and inhibits the expression of amylase. Albumin expression can be seen in some cells. AR42J-13 cells were incubated with 10 nM dexamethasone for 2 weeks (B-D) or 3 weeks (F-H); (A) Control AR42J-B13 cells were stained for anti-amylase antibodies, the picture showed the overlaid image of fluorescent (FITC) staining and the transmit image. (B-D). After 2 weeks of Dex treatment, cells were fixed and stained for anti-amylase antibodies (B). (C). The transmit image of (B). (D). The overlay image of (B) and (C). Arrow cells were extremely flattened and the expression of amylase was almost gone. (E). Control AR42J-B13 cells were stained for anti-albumin antibodies, the picture showed the overlaid image of fluorescent (FITC) staining and the transmit image. (F-H). After 3 weeks of Dex treatment, cells were fixed and stained for anti-albumine antibodies (F). (G). The transmit image of (F). (H). The overlay image of (F) and (G). Some of flat cells strongly expressed albumin, a gene known to be expressed in liver cells.

A





Albumin staining

Fig 3.2 Effect of different concentrations of Dex on the growth rate, morphology change and the expression of albumin. (A) Cell growth was analysed by seed $1x10^5$ cells in 35mm dishes with different concentrations of Dex (0, 1, 10, 100, 1000 nM). Cells were counted after 7 days of Dex treatment. Values are expressed as cell number ($x10^4$) and are means ±SEM of triplicate determinations from an individual experiment. (B-F) Cells were incubated without or with dexamethasone (0, 1, 10, 100, 1000 nM) for 2 weeks, and then fixed and stained for albumin. (G) Every other day, RU486 was added at 2.5 μM, the treatment commencing 1 hour before addition of 1 μM Dex. After 14-days treatment, cells were fixed and stained for albumin.

3.3 Characteristics of pancreatic hepatocytes

In order to determine whether these cells express hepatocytic markers, the Dex-treated cultures were tested for expression of eight liver-specific markers including transferrin (TFN), α1-antitrypsin (α1-AT), transthyretin (TTR), glucose-6-phosphatase (G6Pase), and α-fetoprotein (AFP) (Fig 3.3A-E). At least some of the flattened cells show expression of all these liver markers. Interestingly, when cultured with 10 nM Dex for 6 weeks, some of these cells tended to form bile canaliculi-like structures, and P-glycoprotein (MDR) was localised in the membrane surrounding the apical surface of hepatocytes (Fig 3.2H). Moreover, we also found phenol sulphotransferase (PST) and dehydroepaindrosterone sulphotransferas (DHEAST) were expressed, enzymes having a widespread distribution in adult rat livers (Tosh et al., 1996) (Fig.3.3F-G).

The flattened cells were then also examined by using electron microscopy, the results also showed typical features of hepatocytes such as extensive endoplasmic reticulum, numerous mitochondria and structures resembling bile canaliculi (Shen et al., 2000; experiments performed by Dr. Tosh, data not shown). Additionally, to determine whether or not mitochondria were functional int these cells containing numerous mitochondria, they were loaded with 1 μM Dihydrorhodamine 123 at room temperature. After 15 min, the fluorescent signals were only observed in the flattened cells indicating these hepatocytes contained functional mitochondria (Fig 3.4).

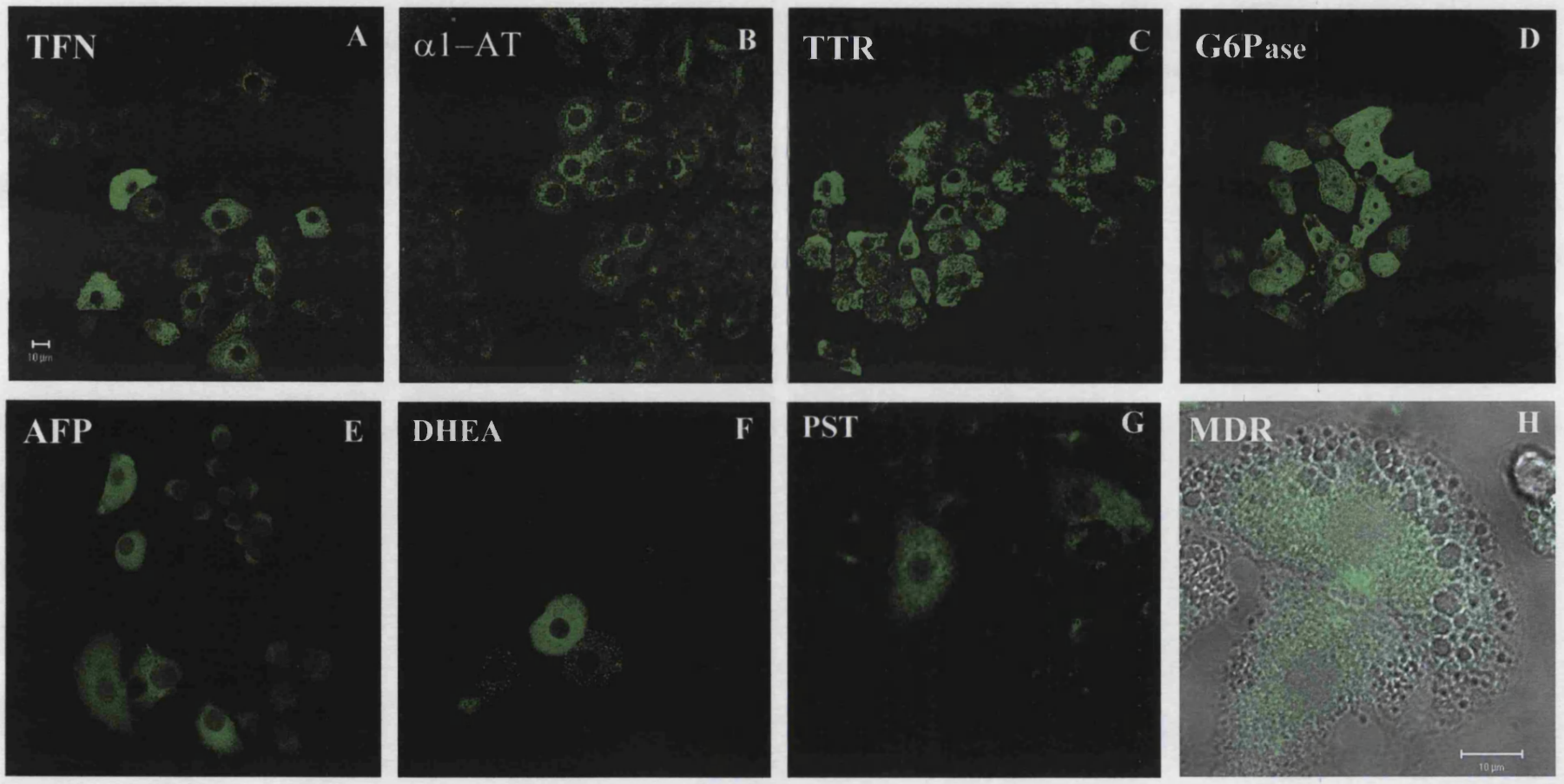

TFN
A
10 μm
α1–AT
B
TTR
C
G6Pase
D
AFP
E
DHEA
F
PST
G
MDR
H
10 μm

Fig 3.3 Dexamethasone treatment results in the expression of liver markers. Cells were incubated with 10 nM dexamethasone for 2 weeks (A-C), 3 weeks (D-E), 6 weeks (F-H) and stained with (A) transferrin (TFN) (B) α_1-antitrypsin (α_1-AT) (C) transthyretin (TTR) (D) glucose-6-phosphatase (G6Pase) (E) α-fetoprotein (AFP) (F) dehydroepaindrosterone sulphotransferas sulphotransferase (DHEA) (G) phenol sulphotransferase (PST) (H) P-glycoprotein (MDR).

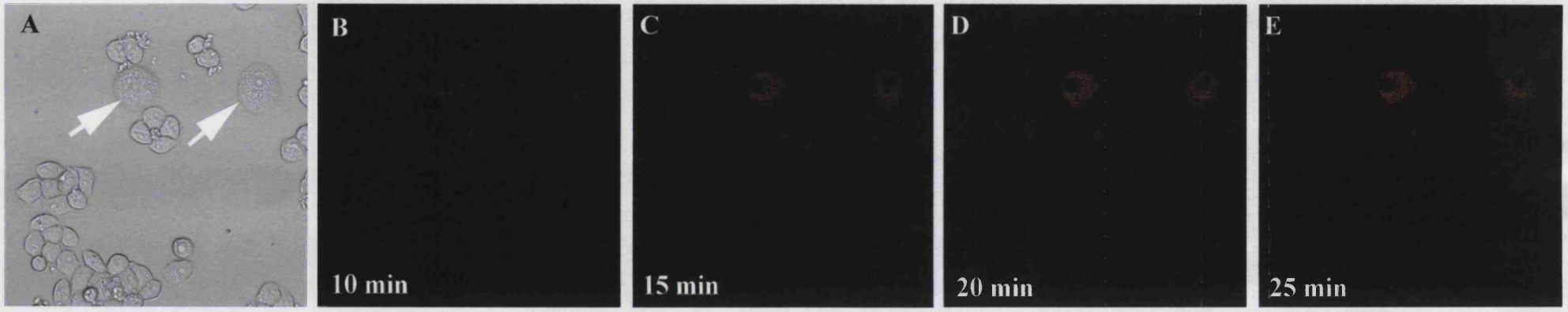

A
B
10 min
C
15 min
D
20 min
E
25 min

Fig 3.4 Mitochondria of flattened cells were stained with Dihydrorhodamine 123. AR42J-B13 cells were treated for 4 weeks with Dex, large cells (>2 fold) were then sorted by cell sorter. After cells were recovered, some cells were seeded on the coverslip, loaded with 1 μM Dihydrorhodamine 123 for 10 min at room temperature, and then fluorescent signals (rhodamin) were observed every 5 minutes by using a Zeiss confocal microscope. (A). Represents the transmit image of (B-E). Arrows indicate the flat cells that converted Dihydrorhodamine to Rhodamin and showed the red fluorescent staining in (B)-(D).

The time course of the transdifferentiation process was studied with 1 μM Dex. The various liver markers appeared in sequence (Fig 3.5). Some cells become flattened by 5 days and some of these began to express transferrin and G6Pase. Albumin starts to appear from 9 days, only in those cells already expressing G6Pase (Fig.3.5; Fig 3.6D). Double staining for amylase and one of the liver markers showed that the cells expressing liver markers did not express amylase (Fig. 3.6F-H), although a few cells were seen with a faint co-expression, as might be expected if the hepatocytes had formerly been exocrine cells.

Because it was expressed in a large number of cells and was one of the earliest differentiation markers to appear (see below), we used G6Pase to score several experiments. G6Pase is normally found in the β cells of the pancreas as well as in liver, but no insulin positive cells were found either in the control or in the Dex-treated AR42J-B13 cells, so we regard G6Pase as a valid marker for hepatocytes in this series of experiments. The proportion of G6Pase positive cells increased to about 25% and that of albumin positive cells to about 6% by 2 weeks in 1 μM Dex (Fig 3.6K).

When cells are stained with two liver markers, the later marker is found only in cells expressing the early marker, for example many cells expressing G6Pase also express transferrin and many expressing transferrin also express albumin. These results suggest the transdifferentiation is a progressive process. Initially, the cells lose the pancreatic phenotype, then they start to express some hepatic genes like G6Pase, α-fetoprotein, transferrin, and then later they express larger amounts of albumin and other gene products characteristic of differentiated hepatocytes (e.g. phenol sulphotransferase, P-glycoprotein).

Hepatic transdifferentiation is also induced by Dex in the parent line-AR42J (Fig 3.7). Although we also found the formation of albumin expressing cells in the parent cell line AR42J after 2-week treatment, it was at lower frequency than in AR42J-B13. The B13 subclone of cells was used for all experiments reported here.

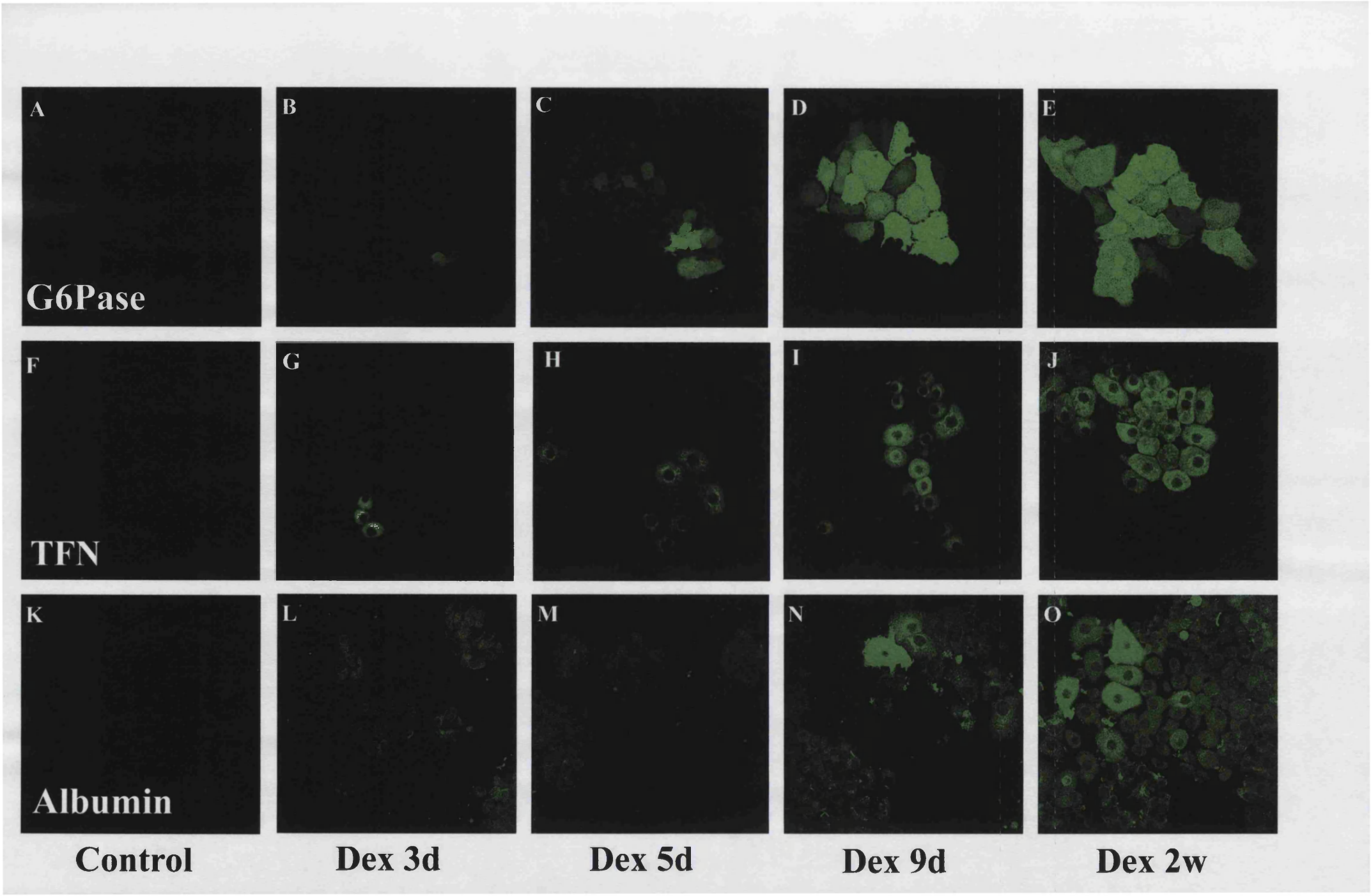
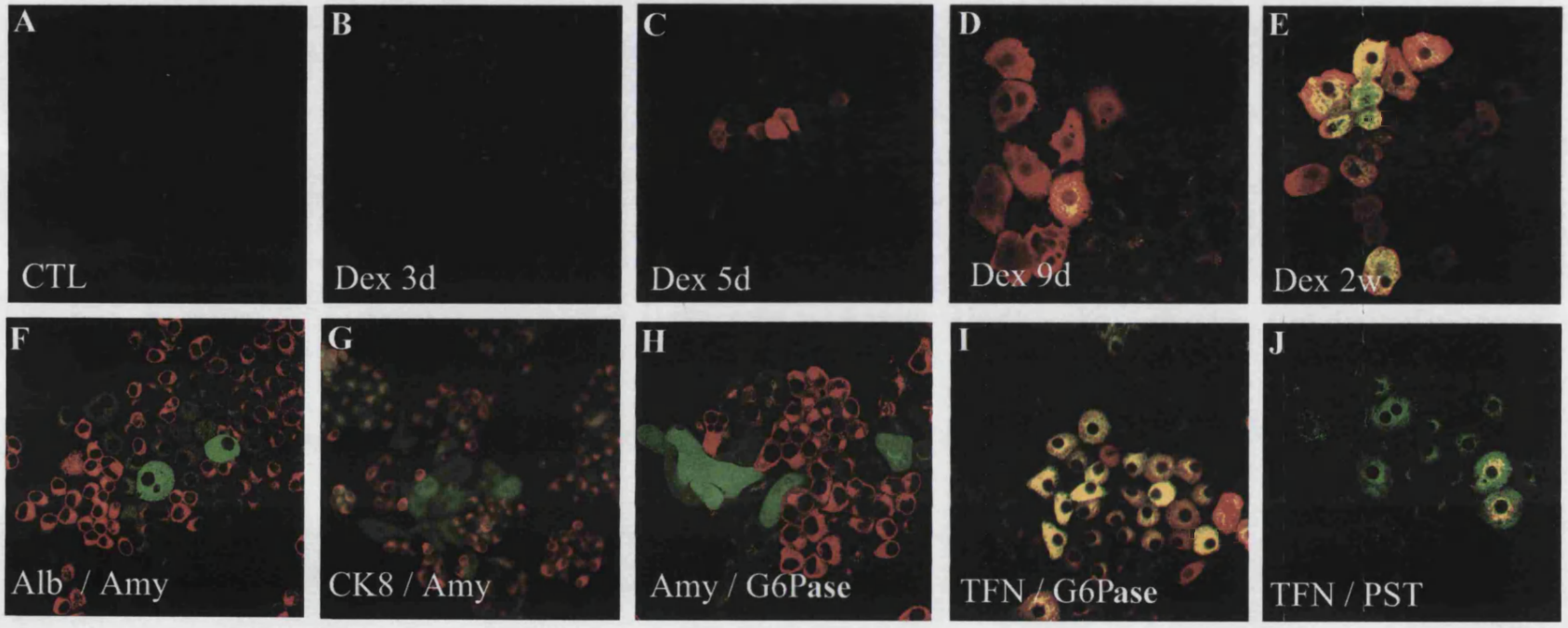

A
B
C
D
E
G6Pase
F
G
H
I
J
TFN
K
L
M
N
O
Albumin
Control
Dex 3d
Dex 5d
Dex 9d
Dex 2w

Fig 3.5 Time-course of the transdifferentiation in AR42J-B13 cells treated with 1 μM Dex. Cells were treated for 0, 3, 5, 9, 14 days and then stained for glucose-6-phosphatase (A-E), transferrin (F-J) and albumin (K-O).


A
CTL
B
Dex 3d
C
Dex 5d
D
Dex 9d
E
Dex 2w
F
Alb / Amy
G
CK8 / Amy
H
Amy / G6Pase
I
TFN / G6Pase
J
TFN / PST

K

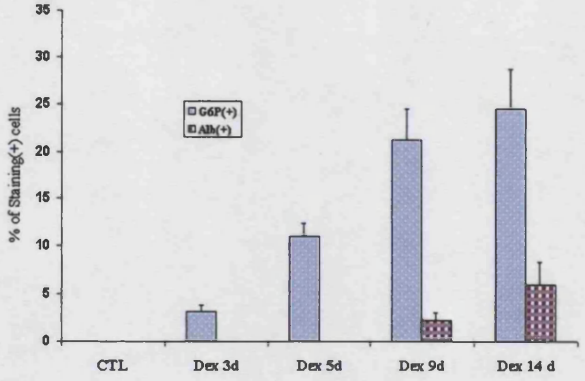
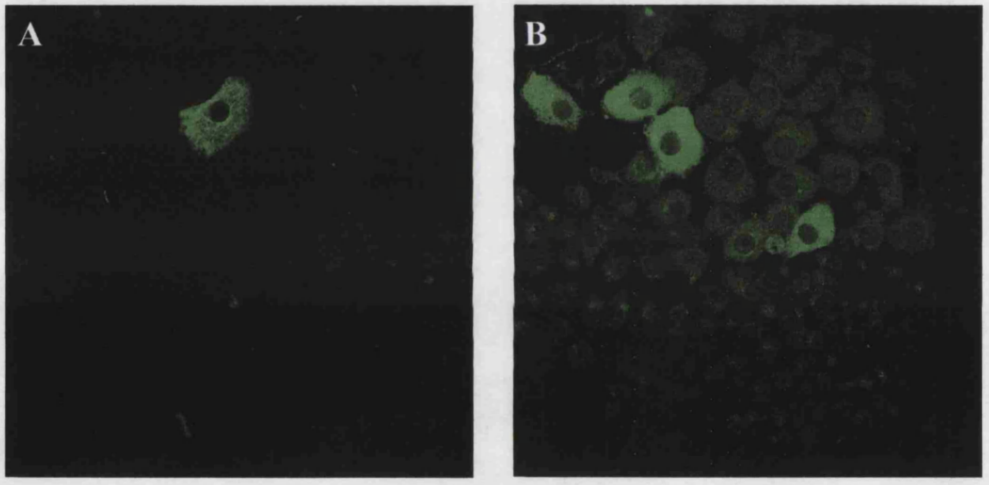

% of Staining(+) cells
35
30
25
20
15
10
5
0
G6P(+)
Alb(+)
CTL
Dex 3d
Dex 5d
Dex 9d
Dex 14 d

Fig 3.6 The transdifferentiation in AR42J-B13 cells treated with 1 μM Dex. (A-E) Cells were treated for 0, 3, 5, 9, 14 days and then dual-immunostained with anti-G6Pase (red) and anti-albumin (green). (F-J) Cells were treated with 1 μM Dex for 2 weeks and double label for (F) anti-amylase (red) and anti-albumin (green) (G) anti-amylase (red) and anti-CK8 (green) (H) anti-amylase (red) and anti-G6Pase (green) (I) anti-G6Pase (red) and anti-TFN (green) (J) anti-PST (red) and anti-TFN (green) at 2 weeks. (K) Time course of appearance of G6Pase and albumin positive cells in 1 μM Dex. Error bars are standard deviations.



AR42j original **AR42J-B13**

Fig 3.7 Dexamethasone treatments lead to the hepatic transdifferentiation in parent cell line AR42J (A) and derivative cell line AR42J-B13 (B). Cells were treated for 14 days with 1 μM Dex and then stained for albumin.

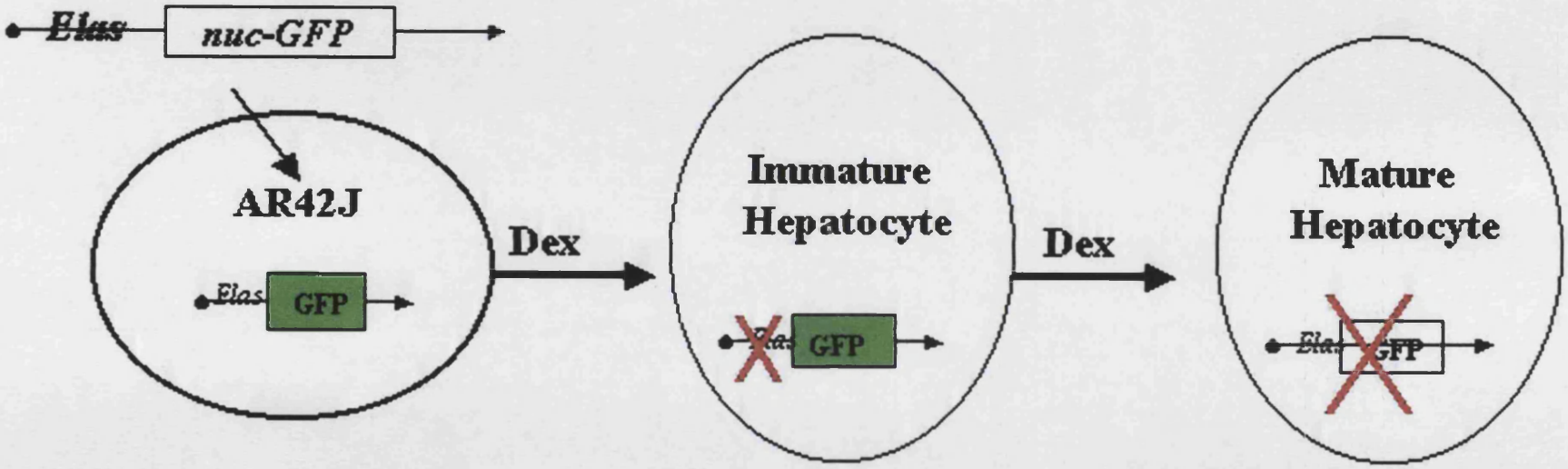
3.4 Origin of pancreatic hepatocytes

Both the parent line AR42J and AR42J-B13 cells are heterogeneous in terms of amylase expression. Although no endocrine cells are present in the cultures in the absence of activin/HGF treatment, there may well be stem cells present that have the potency to produce exocrine, endocrine or hepatocyte-like cells. We only found the conversion of the parent line AR42J cells to insulin-producing cells ((Mashima et al., 1996a; Mashima et al., 1996b) or hepatocyte-like cells was only at low frequency, so we attempted to determine whether the hepatocytes might arise either from differentiated exocrine cells or from stem cells. In order to distinguish these possibilities, we designed a lineage experiment based on the perdurance of green fluorescent protein. Wild type GFP protein is more stable than many other proteins and can remain in a cell for a number of days after its transcription has been switched off and its mRNA has decayed (Corish and Tyler-Smith, 1999). To distinguish exocrine from undifferentiated cells, we used the 200bp fragment of the elastase promoter to drive GFP (Swift et al, 1989).

We isolated stable transfectants of AR42J-B13 cells containing a nuclear *GFP* gene driven by the elastase promoter. These express GFP in 73% (SD 2.9) of cell nuclei under normal culture conditions where the exocrine phenotype predominates. When Dex is added, it initially enhances the green fluorescence but over several days the proportion of green cells gradually falls. At five days, there are only 29.7% (SD 7.3) of cells that clearly show green nuclei (Fig 3.8B, 3.9E). Of the cells with flattened morphology, associated with the early stages of conversion to hepatocytes, 17.1% (SD 1.7) still have green nuclei (see arrowed cells in Fig 3.8B). 25.1% of cells are found to express G6Pase, and of these cells 38.6% contained both GFP and G6Pase (Fig 3.9). The persistence of the GFP shows that these nascent hepatocytes must have arisen

from cells that formerly had an active elastase promoter and therefore were differentiated exocrine-like cells. We cannot say that all cells losing GFP expression become hepatocytes, nor that all nascent hepatocytes contain residual GFP, but the result does clearly show that the direct transdifferentiation from exocrine cells to hepatocytes is possible. It is also possible that some of the hepatocytes arise from stem cells or other undifferentiated precursors present in the culture.

A


Elas
nuc-GFP
AR42J
Elas
GFP
Dex
Immature
Hepatocyte
GFP
Dex
Mature
Hepatocyte
GFP

B

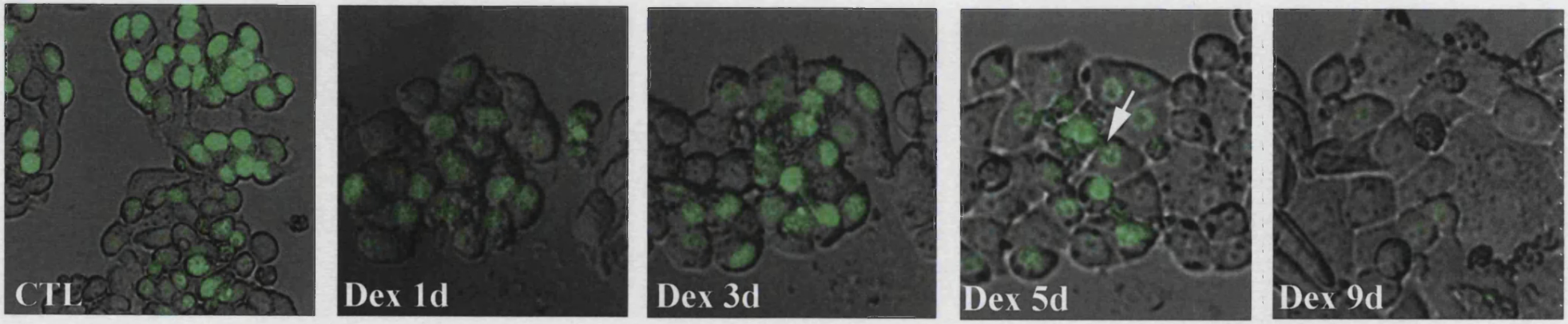
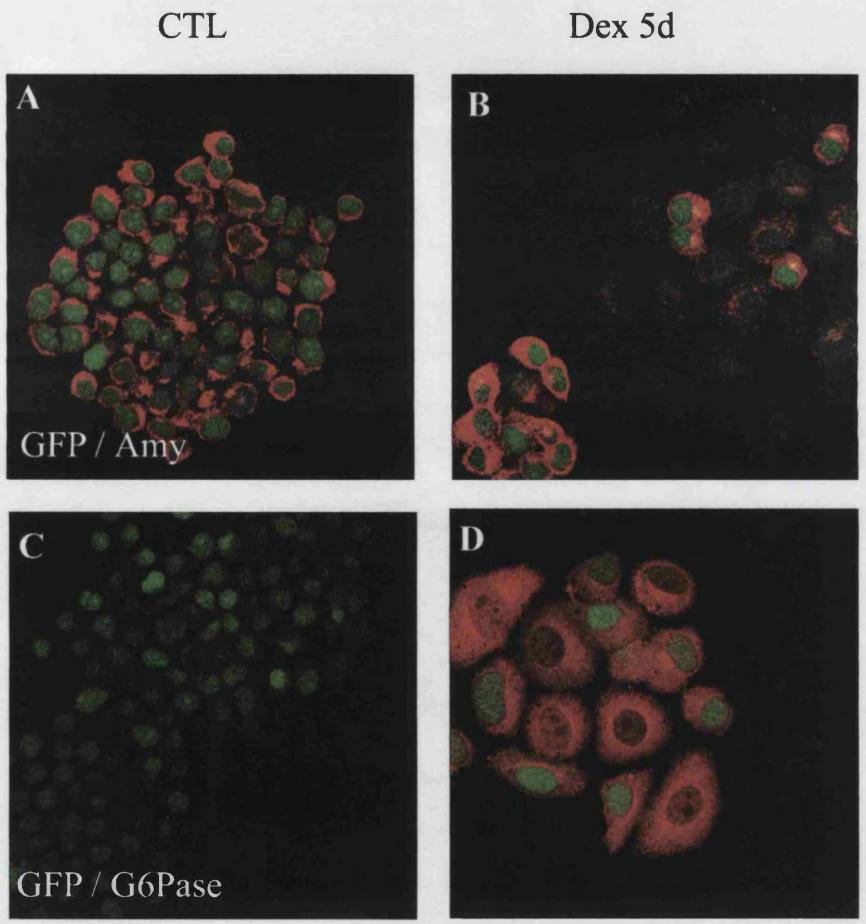

CTL
Dex 1d
Dex 3d
Dex 5d
Dex 9d

Fig3.8 AR42J-B13 cells containing nuclear green fluorescent protein driven by the elastase promoter. (A) Dex treatment caused the metaplastic cells to lose the activation of the Elastase promoter but GFP protein perdures for days. (B) Elas-GFP cells were cultured in medium with 1 μM Dex, and then the same region of the dish was photographed each day with the confocal microscope.


CTL
Dex 5d
A
B
GFP / Amy
C
D
GFP / G6Pase

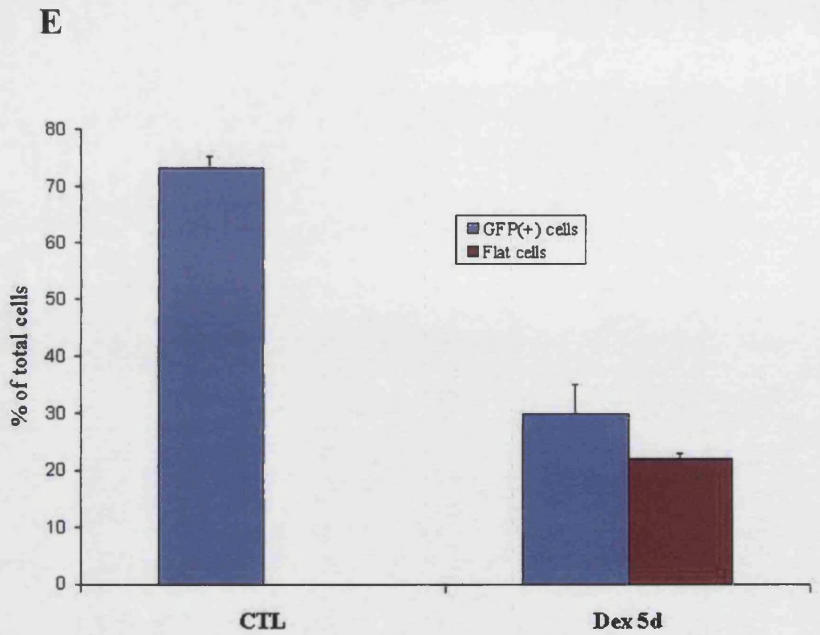
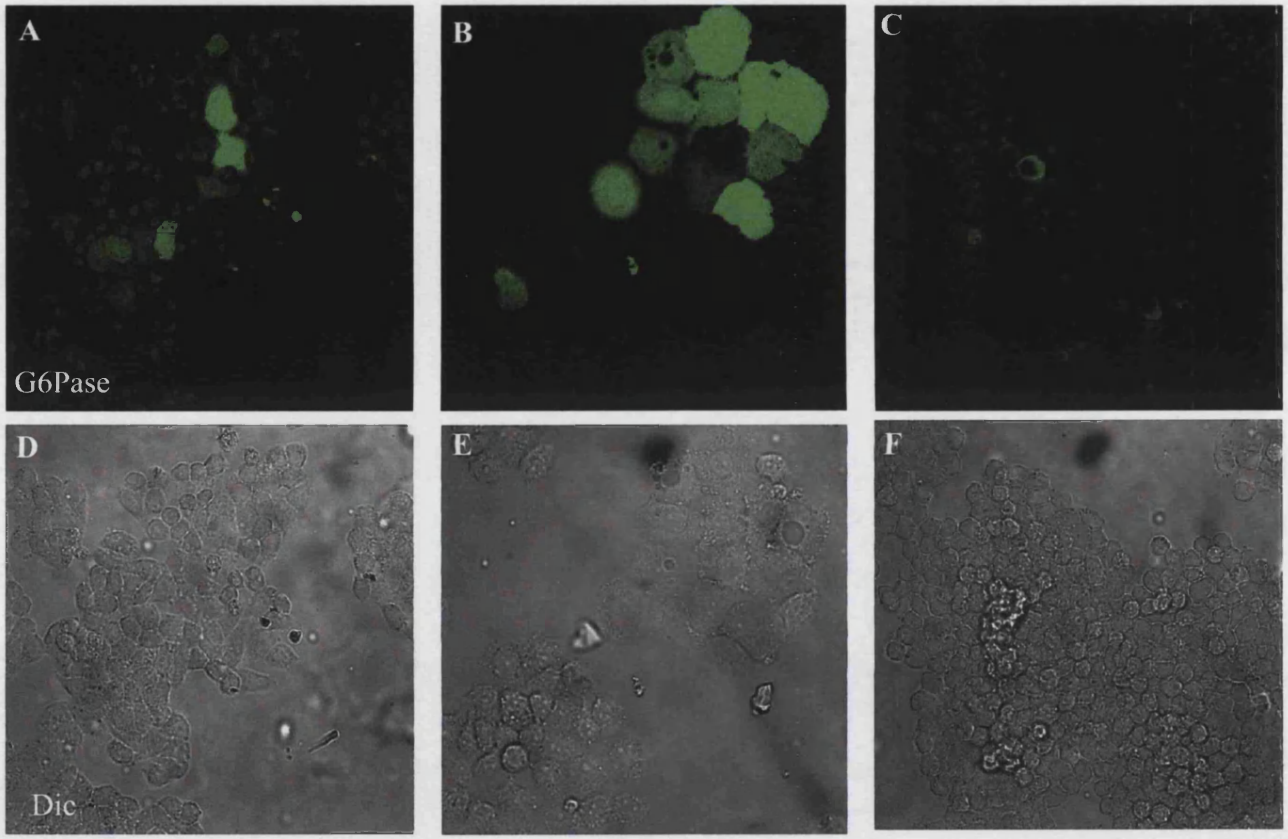

E
% of total cells
80
70
60
50
40
30
20
10
0
GFP(+) cells
Flat cells
CTL
Dex 5d

Fig3.9 AR42J-B13 cells containing nuclear green fluorescent protein driven by the elastase promoter. Cells were incubated with or without 1 μM Dex for 5 days and stained for GFP (green) and (A-B) amylase (red) or (C-D) G6Pase (red). (E). Appearance of GFP positive and flattened cells were counted manually. Total numbers for each specimen are larger than 500. Values are the means±SD for 3 experiments. Error bars are standard deviations.

3.5 Enhancement of the transdifferentiation by combined treatment of Dexamethasone and Hepatocyte growth factor.

Hepatocyte growth factor (HGF), also known as scatter factor (SF) , was originally discovered to play a role in regeneration of the adult liver after partial hepatectomy (Michalopoulos and DeFrances, 1997). HGF is also expressed in the septum transversum mesenchyme that surrounds the developing liver bud, and c-met, the HGF receptor, is expressed on embryonic hepatocytes (Schmidt et al., 1995). Homozygous inactivation of either the HGF or c-met genes yields an embryonic lethal phenotype between days 13 and 16 of gestation, due to hypoproliferation and apoptosis of the hepatic parenchyma (Bladt et al., 1995; Schmidt et al., 1995). These results indicate HGF is a key factor for liver growth.

Mashima has shown that HGF not only can induce AR42J-B13 cells to transdifferentiate into insulin-producing cells, but it can also reduce the effect of dexamethasone on amylase secretion (Mashima et al., 1996b). We tried the combined treatment of Dex and HGF to determine if the transdifferentiation of pancreas to liver can be enhanced by HGF. The result shows that after 5 days of 1 μM Dex and 100pM HGF treatment amylase expression is reduced significantly, and more than 25% of cells were found to express glucose-6-phosphatase. This result suggests that the combination treatment with Dex and HGF can enhance the transdifferentiation process, but there is a large proportion of cells unchanged.


A
B
C
G6Pase
D
E
F
Dic

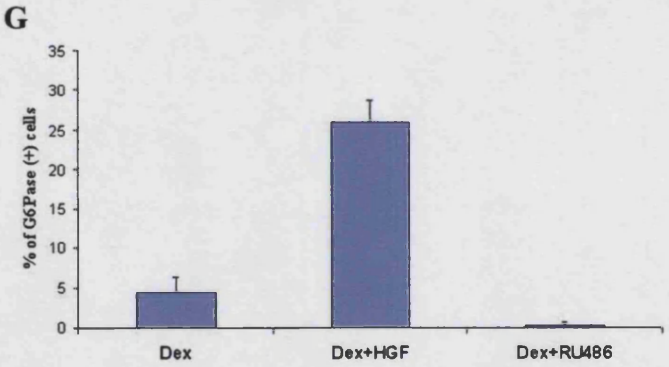

G
% of G6Pase (+) cells
35
30
25
20
15
10
5
0
Dex
Dex+HGF
Dex+RU486

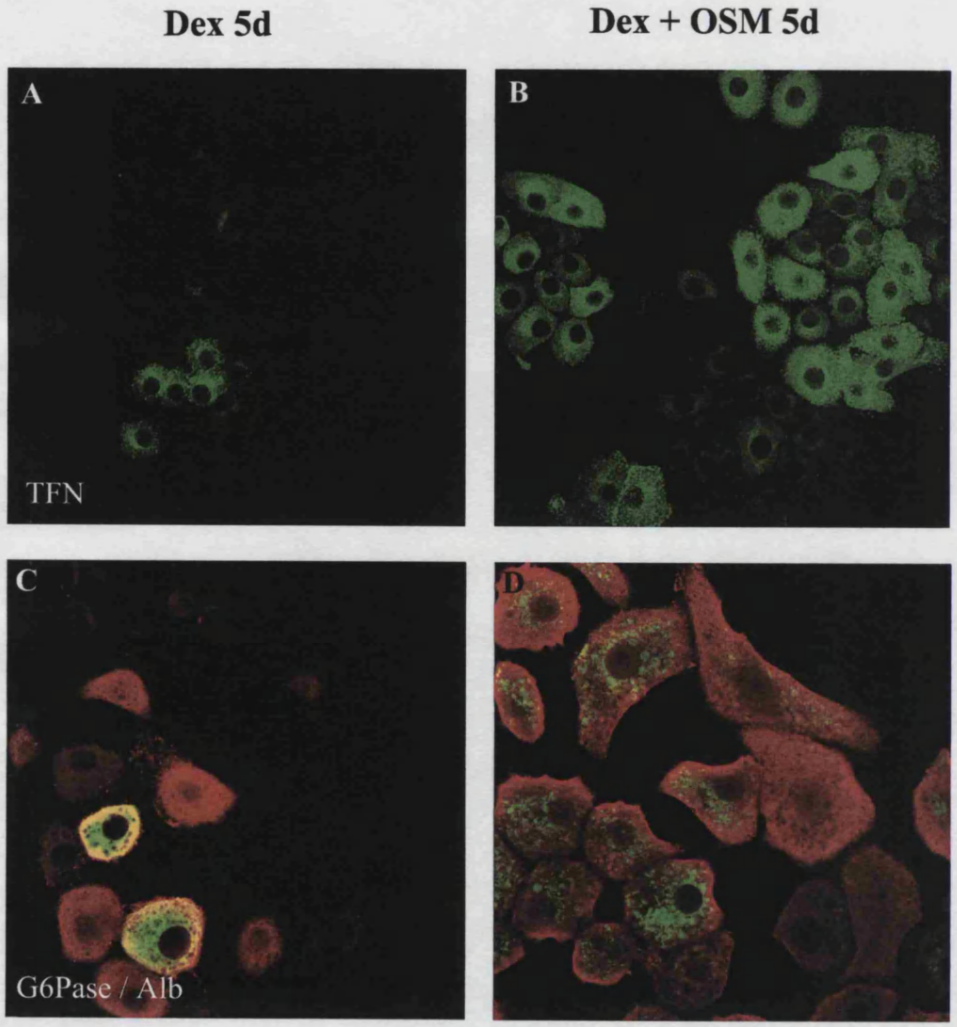
Fig 3.10 The combined treatment of Dex and HGF enhance the proportion of transdifferentiated cells, and pretreatment with RU486 is sufficient to block the conversion. Cells were incubated with 1 μM Dex for 5 days without (A, D) or with 100 pM HGF (B, E). Cells were incubated with 1 μM Dex for 5 days with the pretreatment of 2.5 μM RU486 for 1 hr (C, F). Cells were then stained with anti-G6Pase antibodies. (G) Appearance of G6Pase positive cells was counted from 4 different fields. Total number is >500. Error bars are standard deviations.

3.6 Almost total conversion of AR42J-B13 cells to hepatocytes by combined treatment of Dexamethasone and Oncostatin M

Although the combined treatment of Dex and HGF can enhance the transdifferentiation process, there is still a large proportion of cells unchanged. Here, we also added Oncostatin M (OSM) to the culture to examine if we can convert most cells to hepatocytes in the presence of Dex. Oncostatin M (OSM) is a member of the interleukin-6 (IL-6)-related cytokine family that includes IL-6, IL-11, leukemia inhibitory factor (LIF), ciliary neurotrophic factor and cardiotrophin-1. These cytokines often exhibit similar functions since their receptors utilise gp130 as a common signal transducer (reviewed in Taga and Kishimoto, 1997). Miyajima and colleagues provided evidence to demonstrate that OSM, in combination with glucocorticoid, can induces maturation of fetal hepatocytes *in vitro* (Kaymiya et al., 1999). Furthermore, hematopoietic cells expanding in the fetal liver produce OSM and studies of gp130 knockout mice reveal an essential role for OSM/gp130 in hepatocyte maturation *in vivo* (Kaymiya et al., 1999).

In particular, two types of human OSM (hOSM) receptors have been identified. hOSM shares many biological functions with LIF mediated by the type I receptor (Malik *et al.*, 1989), such as induction of differentiation in M1 monocytic cells (Rose and Bruce, 1991) and of acute phase proteins in hepatocytes (Richards et al., 1992; Baumann et al., 1993). In contrast, mouse OSM utilises only its specific receptor-type II receptor. As the result, we chose to add human OSM to the culture instead of mouse OSM. 10 ng/ml of human OSM was added along with the 1 μM Dex. The result shows that after 5 days of 1 μM Dex and 100 pM HGF treatment more than 70% of cells were found to express transferrin (Fig 3.11 A-B). The proportion of G6Pase positive cells increased to about 89% and that of albumin positive cells to about 64% by 2 weeks in

1 μM Dex and 10 ng/ml OSM treated cultures. To compare with 25% and 6% in 1 μM Dex treatment, it suggests that the combination treatment with Dex and OSM can cause almost total conversion of AR42J-B13 cells to hepatocytes.


Dex 5d
Dex + OSM 5d
A
B
TFN
C
D
G6Pase / Alb
Dex 2w
Dex + OSM 2w

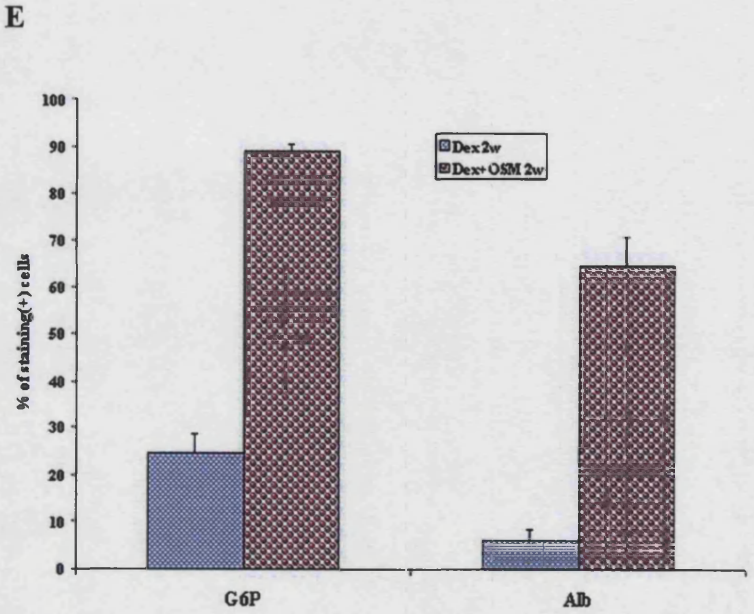

E
% of staining(+) cells
100
90
80
70
60
50
40
30
20
10
0
Dex 2w
Dex+ OSM 2w
G6P
Alb

Fig 3.11 The combined treatment of Dex and OSM convert AR42J-B13 cells to hepatocytes. Cells were incubated with 1 μM Dex for 5 days without (A) or with (B) 10 ng/ml OSM (C-E). Cells were incubated with 1 μM Dex for 14 days without (C) or with (D) 10 ng/ml OSM. Cells were then stained with (A-B) anti-transferrin antibodies or (C-D) dual-immunostained with anti-G6Pase (red) and anti-albumin (green). (E). Appearance of G6Pase and albumin positive cells in 1 μM Dex or 1 μM Dex + 10 ng/ml OSM. Error bars are standard deviations.

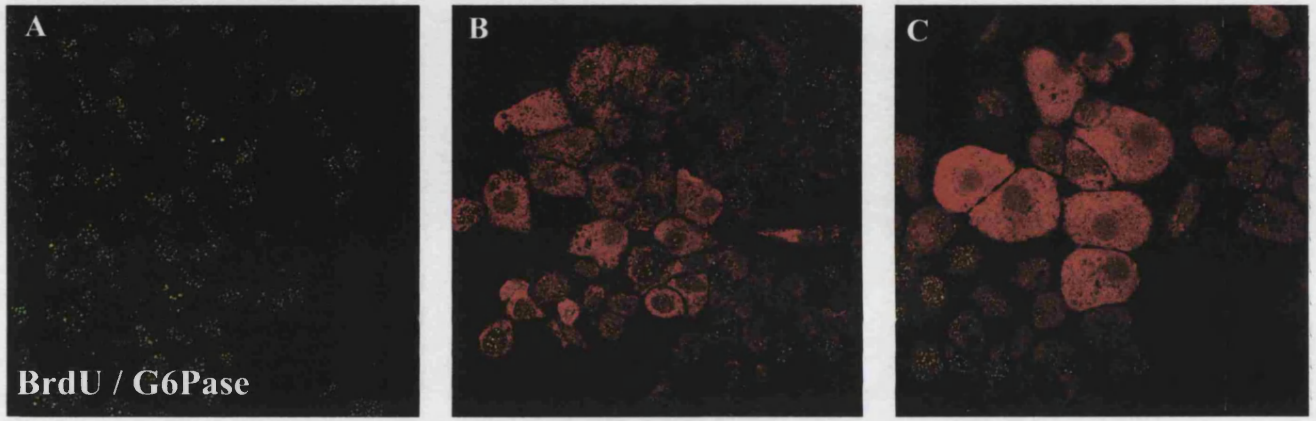
3.7 Transdifferentiation can occur without cell division

The relationship between cell division and cell differentiation is one of the major long-standing problems in development and cell biology. In the classic view - the quantal cell cycle theory (Holtzer et al., 1975), there are two distinct cycles: (1) a proliferative cycle which acts to increase the number of the mother cells, and (2) a unique cell cycle which is responsible to the production of daughter cells different from the mother cells. In many cases of transdifferentiation, cell division and/or DNA replication is essential for transdifferentiation as the progress through cell cycles may cause the alteration of cell state and will cause a stable change in transcription mechanisms in the genome (Okada 1991). For example, in newt lens regeneration from the dorsal iris pigmented epithelium, activation of cell proliferation is a necessary condition for regeneration (Weber et al., 1987). But cell division and/or DNA replication are not essential to all instances of transdifferentiation. Weber also showed that in the transdifferentiation of striated muscle cells of jellyfish to smooth muscle cells can occurs directly without preceding cell division (Weber et al., 1987). Beresford provide a comprehensive list of cases of direct transdifferentiation (Beresford 1990). For example, a single glial progenitor cell can be converted to the oligodendrocyte by switch of medium without cell division.

In an independent set of experiments I examined whether cell division was an obligatory step in the transdifferentiation process of AR42J cells to hepatocytes (Fig. 3.7). The cells were labelled with BrdU from the first day of the Dex treatment. At 5 days 51.5% of the cells expressing G6Pase had not incorporated BrdU so cannot have undergone an S-phase during transdifferentiation. The proportion is 68% if oncostatin M is added as well as the Dex. The remainder of the nascent hepatocytes were BrdU-labelled, as were many non-G6Pase expressing cells. Control (untreated) cultures

showed a much higher labelling index due to the increased overall growth rate. This result shows that transdifferentiation to hepatocytes need not involve cell division.

The unlabelled cell count here represents a lower boundary on the proportion of cells transdifferentiating without cell division because some hepatocytes may have undergone an S-phase after the change of cell phenotype. These experiments prove that at least some of the hepatocytes arise by direct transdifferentiation of pancreatic exocrine cells without cell division. Exactly how many cannot be known and we cannot exclude the possibility that some hepatocytes also arise from stem cells or from other non-exocrine cells present in the cultures.



CTL Dex 5d Dex + OSM 5d

D

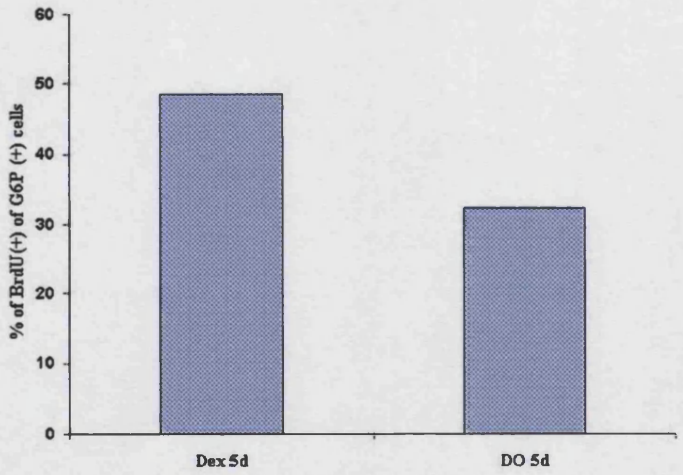


Fig 3.12 Transdifferentiation to hepatocytes need not involve cell division. (A-C) Cells were incubated without or with 1 μM Dex and 10 ng/ml OSM for 5 days, continuous BrdU label from day1, stained at day 5 with anti-G6Pase (red) and anti-BrdU (green). Many G6Pase positive cells have not incorporated BrdU. (D) Appearance of BrdU labeled cells in G6Pase expressing cells. Error bars are standard deviations.

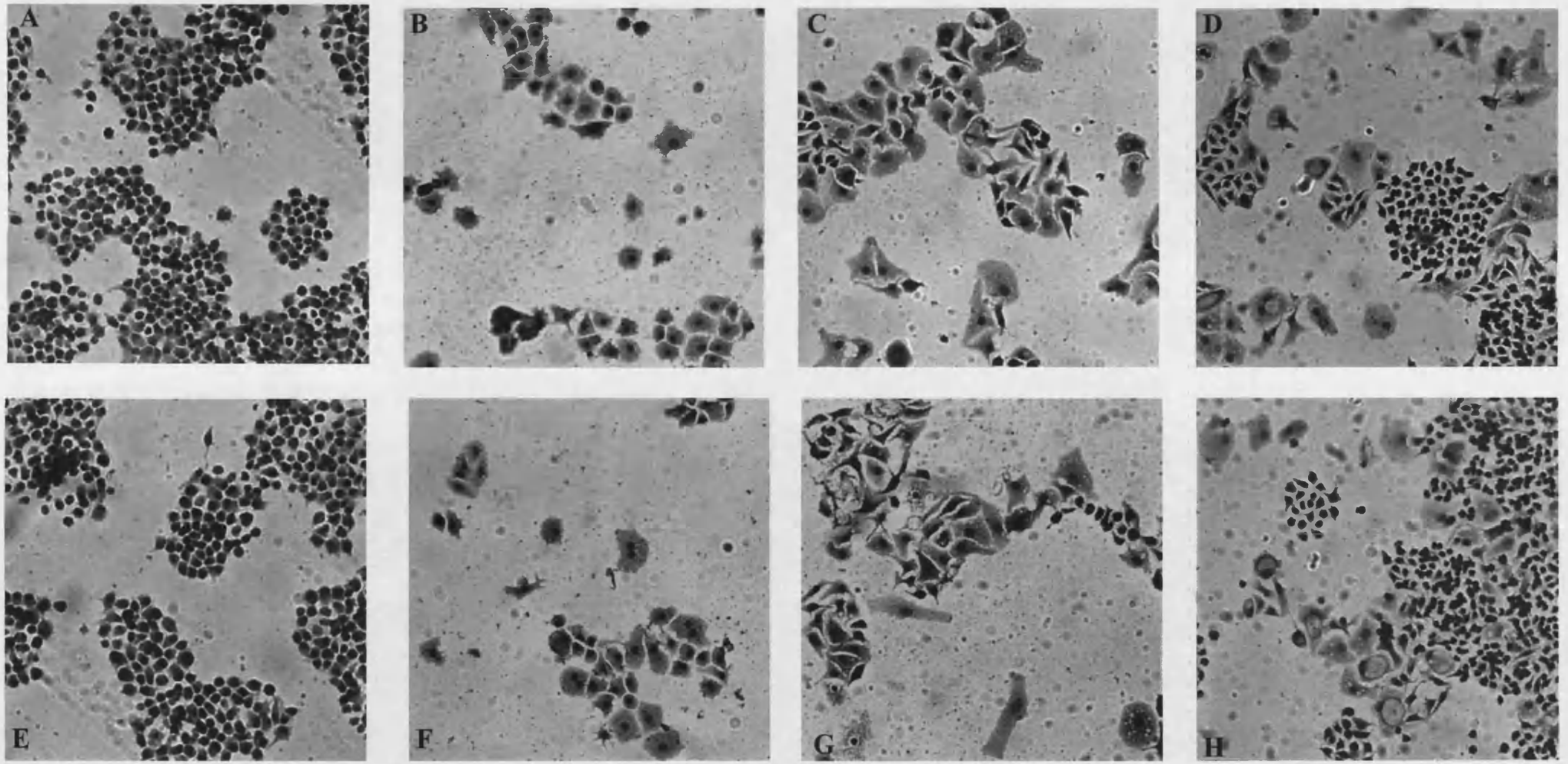
3.8 Determination of the stability of pancreatic hepatocytes

Basically, metaplasia (or transdifferentiation) is a unidirectional process from cell type A to B. Reversible change from B to A is rare. More precisely, the reversible change is outside the definition of transdifferentiation (Okada 1991). In order to determine whether or not the transdifferentiation is a unidirectional process, I investigated the stability of the pancreatic hepatocytes.

AR42J-B13 cells were treated for 2 weeks with 1 μM Dex, then split and cultured with 1 μM Dex and 10 ng/ml OSM for further 4 days to covert the cells into hepatocytes. Cells were then cultured in control medium without Dex, fixed after 4 days and 10 days, then stained by hematoxylin, anti-transferrin or anti-amylase antibodies. As we can see from Fig 3.13, there are mainly two types of cells in the culture. The untransdifferentiated cells are smaller and the morphology is very similar to control AR42J-B13 cells. In contrast, transdifferentiated cells are larger. Removal of Dex increased the number of untransdifferentiated cells, but there are some transdifferentiated cells present. I further stained those cells with anti-transferrin or anti-amylase antibodies. Basically, we found some of cells expressing transferrin, and plenty of untransdifferentiated cells expressing amylase at a low level (Fig 3.14).

In addition, we tried to use green fluorescence protein driven by the TTR promoter to monitor the change in morphology after removal of Dex. AR42J-B13 cells were treated for 2 weeks with 1 μM Dex, then split and cultured with 1 μM Dex and 10 ng/ml OSM for 4 days prior to transfection with CMV-nucGFP or TTR-nucGFP followed by exposure to 1 μM Dex and 10 ng/ml OSM for a further 72 hrs. Cells were then cultured in control medium without Dex, and the dish was photographed every other day with the confocal microscope. We found some of the cells lost their epithelia-like morphology and the proliferation of the untransdifferentiated cells was

increased, but there were still some cells that can drive TTR promoter to express GFP protein. Although we cannot exclude the possibility that some of the cells dedifferentiated, these results at least suggest that the hepatic transdifferentiation is basically a unidirectional process.



CTL B13

-Dex d0
(Dex2w+DO4d)

-Dex 4d

-Dex 10d

Fig 3.13 AR42J-B13 cells were treated for 2 weeks with 1μM Dex, then split and cultured with 1 μM Dex and 10 ng/ml OSM for further 4 days. Cells were then cultured in control medium without Dex, fixed after 4 days (C, G) and 10 days(D, H), then stained by hematoxylin.

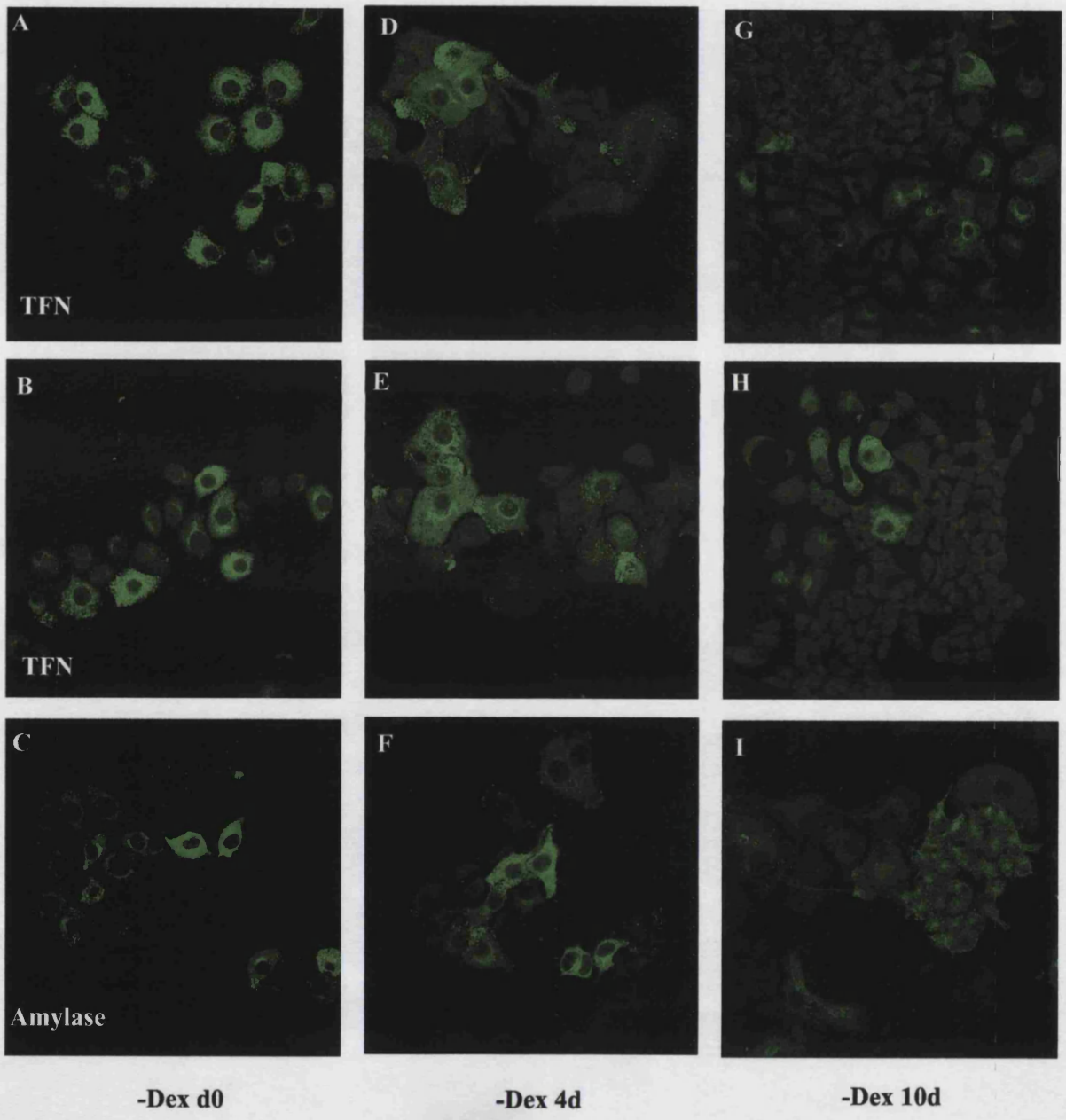
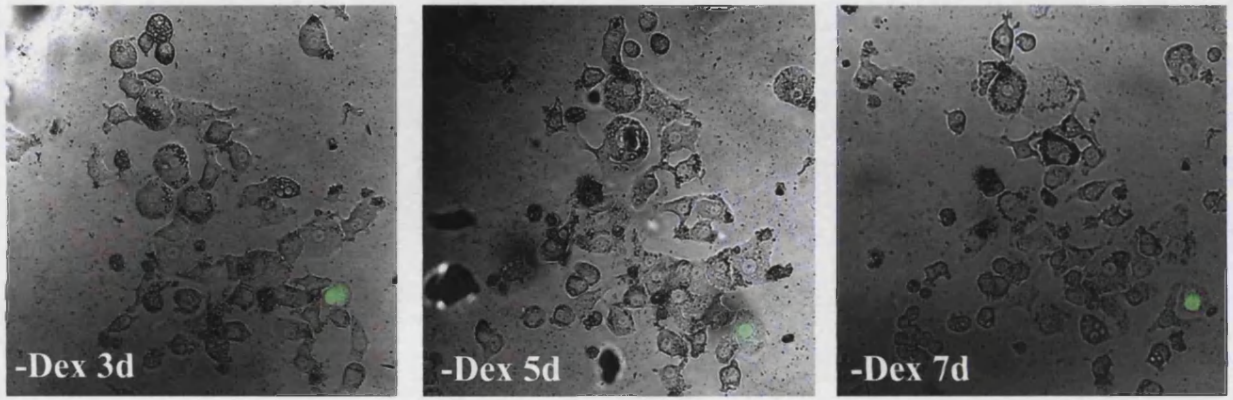

A
TFN
D
G
B
TFN
E
H
C
Amylase
F
I
-Dex d0
-Dex 4d
-Dex 10d

Fig. 3.14 AR42J-B13 cells were treated for 2 weeks with Dex, then split and cultured with 1 μM Dex and 10 ng/ml OSM for further 4 days. Cells were then cultured in control medium without Dex, fixed after 4 days (B, E, H) and 10 days (C, F, I), then stained by anti-transferrin antibodies (A-F) or anti-amylase antibodies (G-I)

CMV-nucGFP



TTR-nucGFP

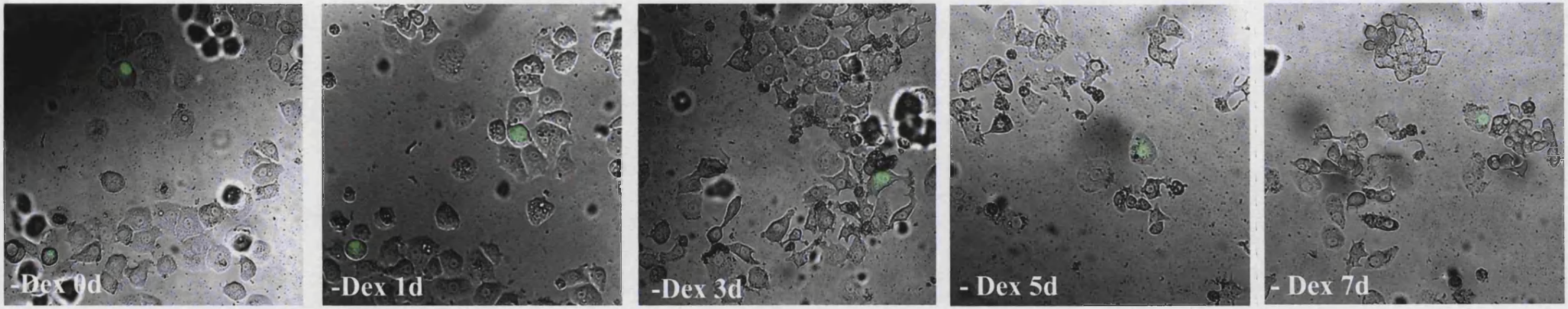


Fig.3.15 AR42J-B13 cells were treated for 2 weeks with 1μM Dex, then split and cultured with 1 μM Dex and 10 ng/ml OSM for 4 days prior to transfection with CMV-nucGFP or TTR-nucGFP followed by exposure to 1 μM Dex and 10 ng/ml OSM for a further 72 hrs. Cells were then cultured in control medium without Dex, and the dish was photographed each other day with the confocal microscope

3.9 Discussion

The work in this chapter demonstrates the ability of the synthetic glucocorticoid dexamethasone to induce the formation of hepatocytes from pancreatic cells. As AR42J cells resemble a pluripotent pancreatic cell, we were interested to establish whether the transformation occurs at the level of a stem cell or of differentiated cells. Although our data cannot exclude a stem cell origin, we do show that at least some of the hepatocytes are formed directly from exocrine-like cells with no intervening cell division, and the hepatic transdifferentiation is basically a unidirectional process. This means that the process fits the definition of a transdifferentiation.

Dexamethasone is a synthetic glucocorticoid with a variety of effects on liver cells including the ability to induce the synthesis of albumin, transferrin, tyrosine aminotransferase, and glucose-6-phosphatase (Chou et al., 1988; Schmoll et al., 1996; Kamiya et al., 1999). It also enhances the differentiation of AR42J cells towards the exocrine phenotype (Logsdon et al., 1985). Our results suggest the possibility that endogenous glucocorticoids may be involved in the normal developmental decisions that operate to subdivide the foregut endoderm into stomach, pancreas, liver and intestine. Although Dex in combination with oncostatin M is known to accelerate the maturation process in fetal murine hepatocytes (Kamiya et al., 1999; Kinoshita *et al.*, 1999), these factors have not previously been considered to be embryonic inducing factors. On the other hand, fibroblast growth factors have been implicated in the primary induction of the liver (Jung et al., 1999), and have also been shown able to provoke hepatic metaplasia of the pancreas (Krakowski et al., 1999). It remains to be seen whether FGFs activate the same genetic pathway to induce hepatic differentiation.

In a parallel experimental project, we started to use FGF treatment to trigger the transdifferentiation, but with limited success.

In sequences of elastase-GFP cells photographed on successive days it is possible to see a direct transformation of exocrine cells into flat cells. And we have shown that GFP can perdure from cells with an active elastase promoter into flattened cells expressing G6Pase. It indicates that at least some of the hepatocytes are formed directly from exocrine-like cells. This result may be compared to the observations from animal models which suggest hepatocytes might derive either from exocrine (Reddy et al., 1984), endocrine (Krakowski et al., 1999) or ductular cells (Makino et al., 1990). Because of the possibility of additional lineage pathways, we have examined whether other types of cells could be converted to hepatocytes. We have treated Rin-5F (rat pancreatic β cells), Capan-1 or PANC-1 (both human pancreatic duct cells) with Dexamethasone and/or Oncostatin M. These treatments were without effect although Dex treatment did cause the reduction of pancreatic phenotype on RIN-5F cells (data now shown, also see Chapter 6.4). Although we can not exclude the possibility that other type of pancreatic cells can transdifferentiate into hepatocytes, these negative results may suggest that glucocorticoid or the glucocorticoid signalling pathway is not sufficient to trigger the hepatic metaplasia from non-exocrine cells. Additional support for the exocrine-hepatocyte transdifferentiation is provided from the two cases of reverse conversion of hepatocyte to pancreas: (1) exocrine pancreatic tissue in the liver of rat induced by polychlorinated biphenyls (Rao et al., 1986a); (2) Exocrine pancreatic tissue in the liver of a 41-year-old patient with posthepatitic cirrhosis (Wolf et al., 1990). Both examples reflect the close relationship between pancreatic exocrine cells and hepatocytes. In the later chapters, I shall describe the use

of the AR42J-B13 cells to establish the molecular mechanism involved in the transdifferentiation.

CHAPTER 4

MOLECULAR BASIS OF TRANSDIFFERENTIATION OF PANCREAS TO LIVER

4.1 Introduction

Liver and pancreas originate from the foregut endoderm (Zaret, 1998; Slack, 1995). FGFs have been shown to be implicated in the specification of liver development (Jung et al., 1999), but the mechanism, which regulates the endodermal epithelium to develop into pancreas and liver, remains unclear. We presume there might be a gene(s) whose expression distinguishes these two tissues in normal development. Under certain conditions, a discrete change in this gene expression might cause the hepatic metaplasia of pancreas in postnatal life (see chapter 1). As a result, the system of hepatic metaplasia of pancreas provides a novel opportunity to elucidate the cellular and molecular mechanisms that control the development of two important endodermal organs- liver and pancreas.

Although hepatic metaplasia of the pancreas is well studied, there were only a few studies (Rao et al., 1989; Dabeva et al., 1995) to investigate the genes that are involved in the transdifferentiation. They both isolated the ductal epithelial progenitors from copper depletion-repletion (CuD) rats, and used the cells to investigate the mRNA level of liver enriched factors (LETFs). Their results show these cells express HNF-1, HNF-3α, HNF-3β, HNF-4, and members of the CCAAT/enhancer binding protein (C/EBP) family. However, as I described in Chapter 1.5, some of these transcription factors are also normally found to be present in the pancreas and other tissues (reviewed in Cereghini, 1996; Fig 4.1), so these results were not sufficient to understand the conversion of pancreatic cells to hepatocytes.

Liver-specific gene expression in adult hepatocytes relies on five families of evolutionary conserved transcription factors that are liver-enriched but not restricted to this tissue. They are hepatocyte nuclear factors 1, 3, 4, 6 (HNF-1, HNF-3, HNF-4, HNF-6) and CCAAT/enchancer-binding protein (C/EBP). These factors function in

unique combinations (Tronche and Yaniv, 1992; Simone and Cortese, 1992; Cereghini, 1996; Samadani and Costa, 1996; Darlington 1999), often synergistically, to stimulate cell-specific transcription. Each family is composed of several members displaying similar, if not identical, DNA recognition properties and sharing structural homology in their DNA binding domains. The homo- and heterodimerization between members of a particular transcription factor family adds an additional level of complexity in gene regulation.

We believe at least part of the process of metaplasia is governed by the combined action of the LETFs as they are actually involve in regulating many liver specific genes (Simone and Cortese, 1992). For example, Fig 4.2 shows the promoter region of *albumin* and *transthyretin* contained the binding site of HNF-1, HNF-3, HNF-4, and C/EBP. In order to determine how the combined actions of the LETFs to regulate the transdifferentiation process and/or liver differentiation, I have investigated the expression of LETFs in the transdifferentiation and also determine the importance of the factors in the process.

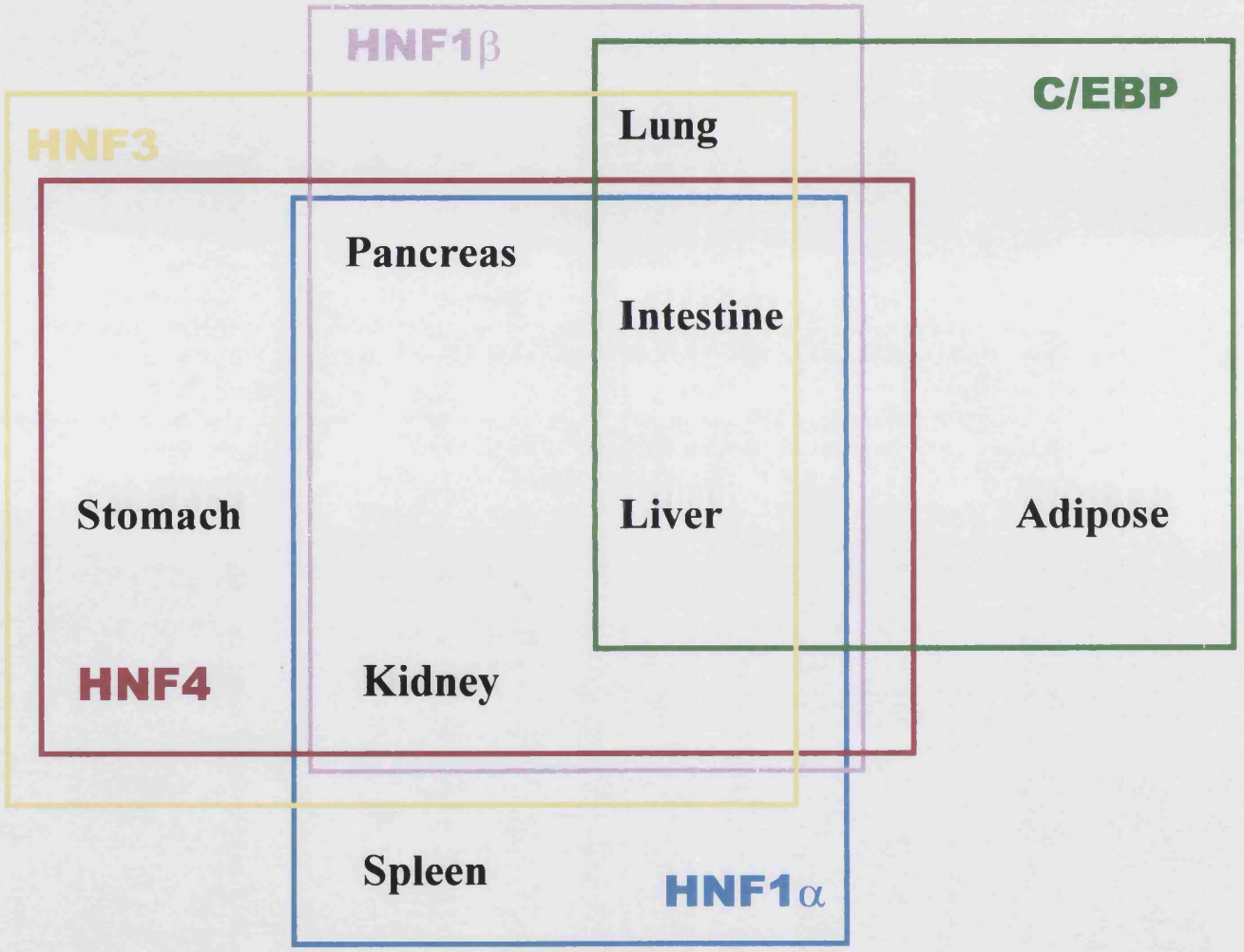

HNF1β
C/EBP
HNF3
Lung
Pancreas
Intestine
Stomach
Liver
Adipose
HNF4
Kidney
Spleen
HNF1α

Fig 4.1 Schematic representation of expression for liver enriched transcription factors in the different organs. The figure was modified from Tronche and Yaniv 1992; Cereghini 1996; Lekstrom-Himes and Xanthopoulos 1998.

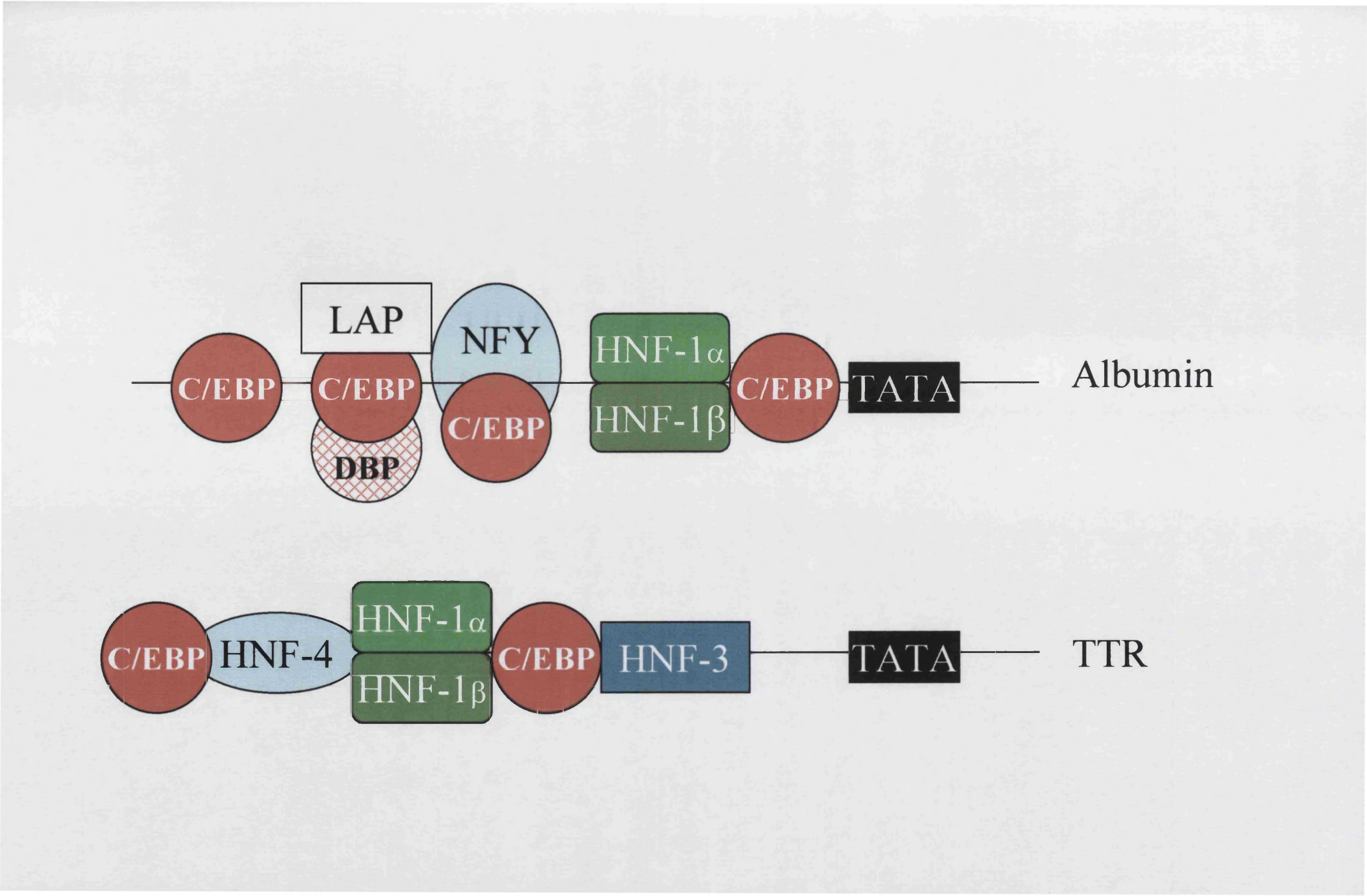

LAP
NFY
HNF-1α
C/EBP
C/EBP
C/EBP
C/EBP
HNF-1β
TATA
Albumin
DBP
C/EBP
HNF-4
HNF-1α
C/EBP
HNF-3
TATA
TTR
HNF-1β

Fig 4.2 A diagram of the interactions between cis-acting elements and trans-acting factors in the promoters of *albumin* and *transthyretin* (TTR). The figure was modified from Simone and Cortese 1992.

4.2 Determination of genes involved in the conversion of pancreas to liver

In order to probe the molecular mechanism of the transdifferentiation process, we examined the expression of various transcription factors associated with hepatic differentiation. Our attention was initially drawn to molecules commonly regarded as liver transcription factors, namely HNF-1, HNF-3, HNF-4 and HNF-6. All of these factors are normally expressed in the liver, and most of them are also expressed in pancreas. For example, HNF-1, HNF-3β, HNF-4 and HNF-6 are normally expressed in pancreatic β cells. From the results obtained from RT-PCR, I found HNF-1, HNF-3β, HNF-3γ, HNF-4 and HNF-6 are all expressed in AR42J-B13 cells. In comparison with the reduction of the pancreatic transcription factor-p48 (PTF-p48), which regulates the exocrine phenotype, the mRNA levels of these hepatocyte nuclear factors do not increase significantly on Dex treatment. Only the level of HNF-4 mRNA did increase slightly during the transdifferentiation (Fig 4.3).

Hepatocyte nuclear factor 4 (HNF-4), a member of the nuclear receptor superfamily (Sladek et al., 1990), is abundant in adult as well as the fetal liver and intestine, but is present at a lower level in islets of Langerhans and is scarcely detectable in exocrine pancreas (Miquerol et al., 1994). Although the level of HNF-4 mRNA didn't increase significantly, I tried to examine the protein level by immunstaining. I found the staining of HNF-4α increased after adding Dex to the culture (Fig.4.4 F-J). Noticeably, HNF-4α protein begins to translocate into the nucleus after 5 days of treatment with 1 μM Dex. The result suggests that HNF-4 maybe involved in the process of the conversion of pancreas to liver.

CCAAT-enhancer binding proteins (C/EBP) are basic region/leucine zipper (bZIP) transcription factors expressed during the differentiation of adipose tissue and liver. As shown in Fig 4.1, we actually found that C/EBP is not normally expressed in

the pancreas, so I decided to examine the level of C/EBP protein. As expected, we found that C/EBPβ was absent from AR42J-B13 cells, but was induced after just 3d treatment with dexamethasone (Fig.4.4 A-E). Furthermore cells that had activated C/EBPβ also show reduced amylase or increased G6Pase (Fig 4.5A, B). These properties make C/EBPβ a candidate for the master switch regulating the cell phenotype.

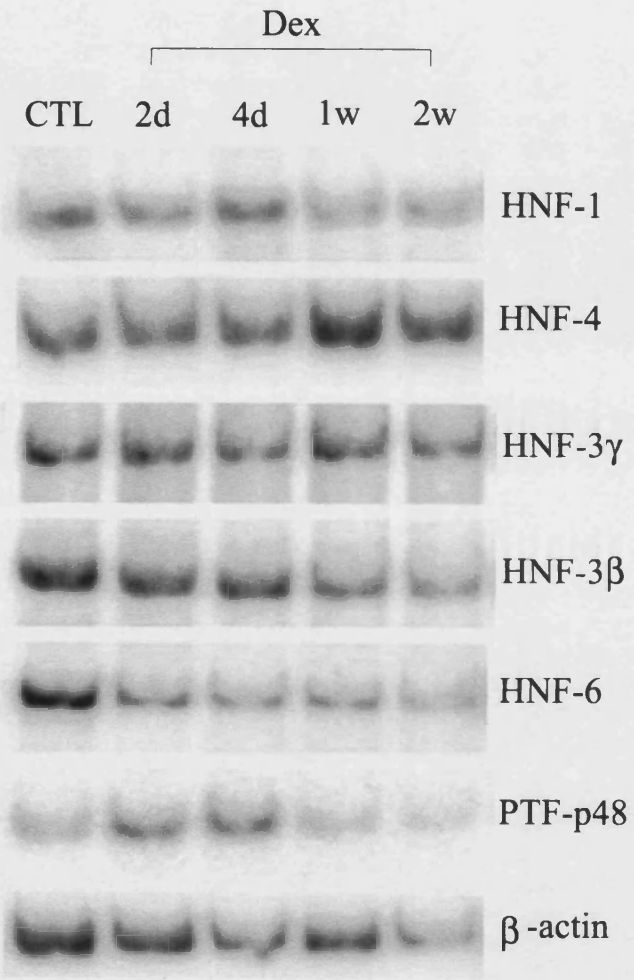
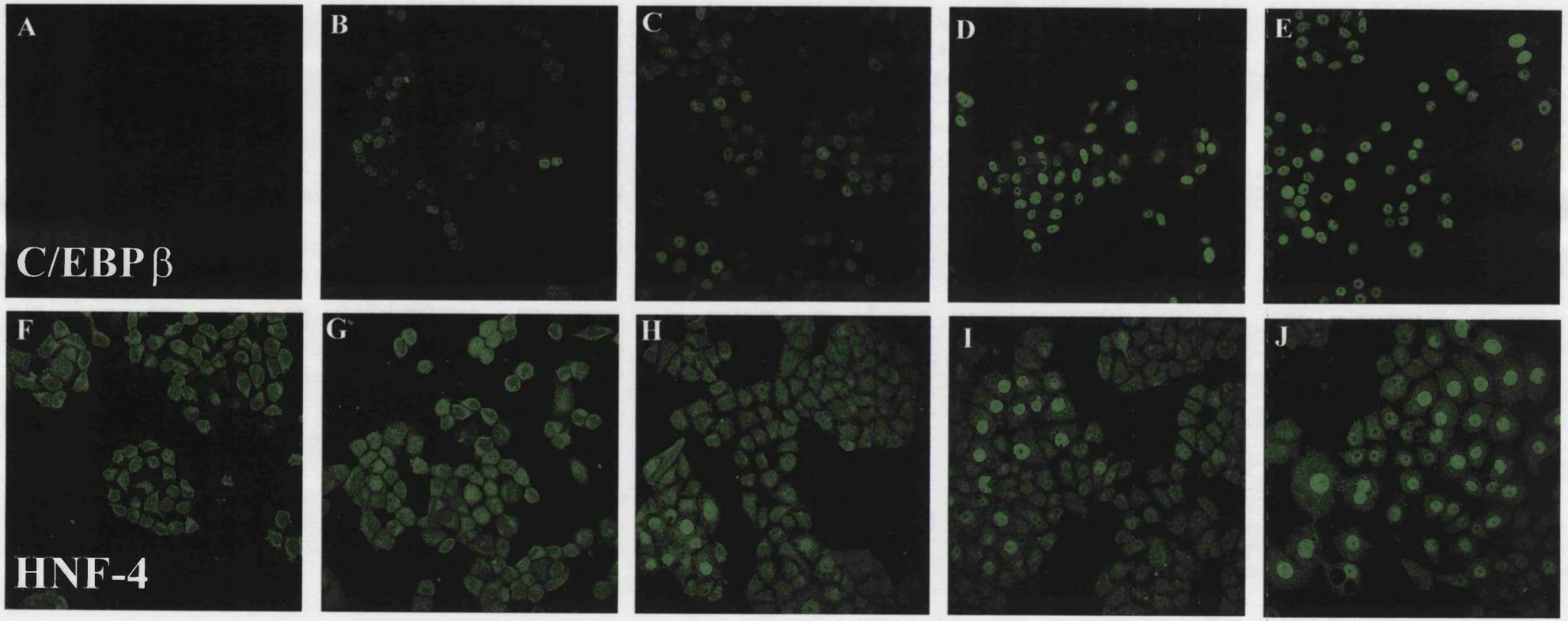

Dex
CTL
2d
4d
1w
2w
HNF-1
HNF-4
HNF-3γ
HNF-3β
HNF-6
PTF-p48
β -actin

Fig 4.3 Time-course of mRNA level of hepatocyte nuclear factors 1, 3, 4, 6 and PTF-p48. Total RNA was extracted from cells incubated with 1 μM Dex for 2, 4 days, 7days, and 14 days, and then mRNA expression was analysed by RT-PCR.


A
B
C
D
E
C/EBPβ
F
G
H
I
J
HNF-4
Control
Dex 3d
Dex 5d
Dex 9d
Dex 2w

4.3 C/EBPβ is a key component of the transdifferentiation process

To determine whether or not C/EBP is a key component of the transdifferentiation process? I decided to transfect AR42J-B13 cells with C/EBPβ, and found that the effects were indeed very similar to those following Dex treatment. In cells expressing the C/EBPβ, amylase fell and liver markers including transferrin, G6Pase and transthyretin became expressed (Fig 4.5E-H). These changes were unaffected by pretreatment with RU486 (Fig 4.6 D, H), which inhibits the Dex effect, showing that C/EBPβ action is downstream of the glucocorticoid receptor activation. While the transfection of LAP, the constitutively active truncated form of C/EBPβ, was also sufficient to cause the transdifferentiation. The result shows that the activation of C/EBPβ is a critical step responsible for switching between pancreatic exocrine and hepatocyte phenotypes. Although transfection of C/EBPβ caused hepatic differentiation, it does not cause the same degree of morphological flattening as the Dex treatment.

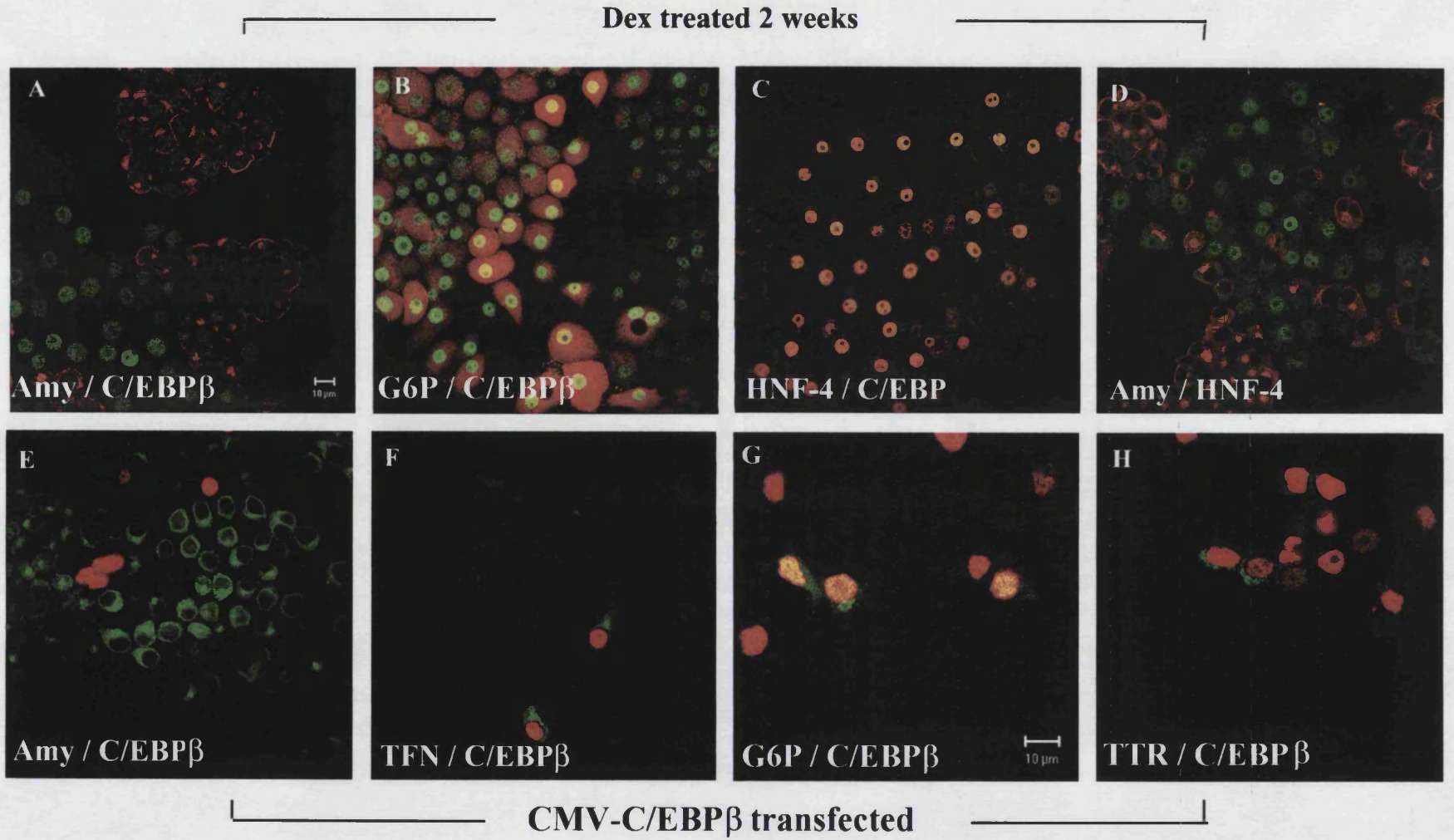

Dex treated 2 weeks
A
Amy / C/EBPβ
10 μm
B
G6P / C/EBPβ
C
HNF-4 / C/EBP
D
Amy / HNF-4
E
Amy / C/EBPβ
F
TFN / C/EBPβ
G
G6P / C/EBPβ
10 μm
H
TTR / C/EBPβ
CMV-C/EBPβ transfected

Fig 4.5 C/EBPβ induces transdifferentiation in AR42J-B13 cells. Cells were incubated with 1 μM dexamethasone for 2 weeks and were then immunostained (A-D).

(A) anti-amylase (red) and anti-C/EBPβ (green)

(B) anti-G6Pase (red) and anti-C/EBPβ (green)

(C) anti-HNF-4α (green) and anti-C/EBPβ (red).

(D) anti-amylase (red) and anti-HNF-4α (green)

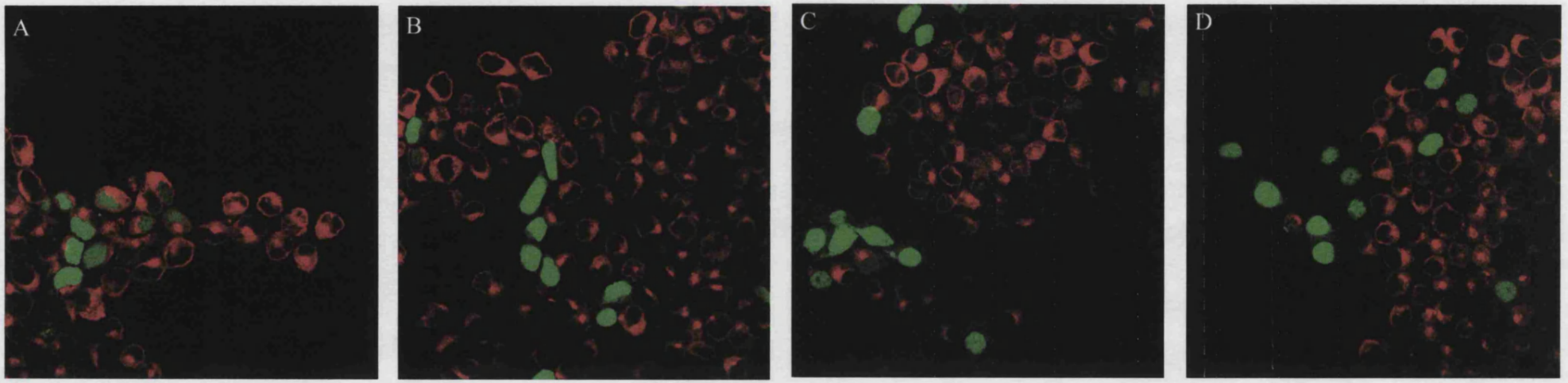
Cells were transfected with pcDNA3-C/EBPβ , and immunostained 4 days after(E-H).

(E) anti-C/EBPβ (red) and anti-amylase (green).

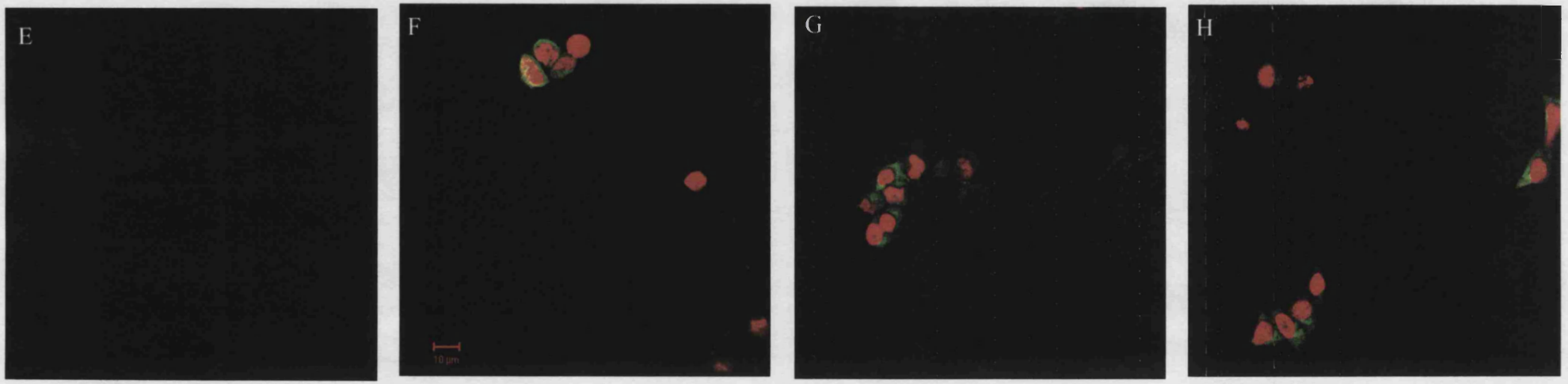
(F) anti-C/EBPβ (red) and TFN (green).

(G) anti-C/EBPβ (red) and anti-G6Pase (green).

(H) anti-C/EBPβ (red) and anti-TTR (green).



Amy/GFP **Amy/LAP** **Amy/C/EBPβ** **Amy/C/EBPβ (+RU486)**



Control **TFN/LAP** **TFN/C/EBPβ** **TFN/C/EBPβ (+RU486)**

Fig 4.6 Overexpression of C/EBPβ caused reduction in the expression of amylase and initiation of the expression of transferrin. AR42J-B13 cells were transfected with pcDNA-nucGFP (A) or pcDNA3 (E) or pcDNA-C/EBPβ (B-D, F-H) without or with 2.5 μM RU486 (D, H). After 4 days of transfection, then stained with (A) Amy (red) + GFP (green) (B-D) Amy (red) + C/EBPβ (green) or (E-H) TFN (green) + C/EBPβ (red)

4.4 Overexpression of LIP inhibits transdifferentiation

C/EBPβ can be transcribed into one mRNA which can then be translated into three isoforms designated C/EBPβ, liver inhibitory protein (LIP) and liver activator protein (LAP) (Descombe and Schibler, 1991). The LIP protein is a 21 kDa molecule which lacks the transactivation domain and acts as a dominant-negative form of C/EBPβ by heterodimerising with the full length C/EBPβ (Darlington 1999). Overexpression of LIP in 3T3-L1 fibroblasts has been shown to inhibit conversion to adipocytes, which is a C/EBPβ-dependent process (Yeh et al., 1995).

To determine whether C/EBPβ is an essential requirement for the transdifferentiation, I transfected LIP into AR42J-B13 cells under different culture conditions. CMV-LIP was transfected into AR42J-B13 cells and 24hrs later they were induced to undergo transdifferentiation by treatment of 1 μM Dex and 10 ng/ml OSM. This treatment can induce more than 60% of AR42J-B13 cells to express G6Pase, TFN and TTR within 3 days. After 4 days of treatment the cells were stained for C/EBPβ (or LIP) and G6Pase, TTR or TFN (Fig 4.7 A-L). The C/EBPβ antibody used in this experiment was raised to the C-terminus, so it can recognise both C/EBPβ and LIP. In all cells in which the LIP was present as determined from the more intense nuclear staining (Fig 4.7. C, G, K), the liver marker was absent, showing that overexpression of LIP is sufficient to prevent the transdifferentiation and suggests that C/EBPβ is required for the conversion.

To investigate whether C/EBPβ is necessary not only for induction, but also for maintenance of the hepatic phenotype I treated AR42J-B13 cells with Dex for 8-10 weeks, and then introduced LIP. In order to ensure that all cells were converted to hepatocytes, I pretreated the cells with 1 μM Dex and 10ng/ml OSM for 5 days, then

transfected the cells with CMV-LIP and kept them in the medium containing 1 μM Dex and 10ng/ml OSM for an additional 72hrs. Almost all of these cells express liver markers (G6Pase, TFN, or TTR), but the LIP positive cells lacked any of the liver markers compared to untransfected cells, showing that an existing hepatic phenotype can be inhibited by LIP (Fig 4.8 C, G, K). In cells transfected with control CMV-nuclear GFP, the level of liver marker expression was similar to the untransfected cells indicating that transfection itself was not responsible for preventing or inhibiting the markers expression (Fig 4.7 D, H, L; Fig 4.8 D, H, L). The results also indicate that C/EBPβ is essential for maintaining the hepatic phenotype.

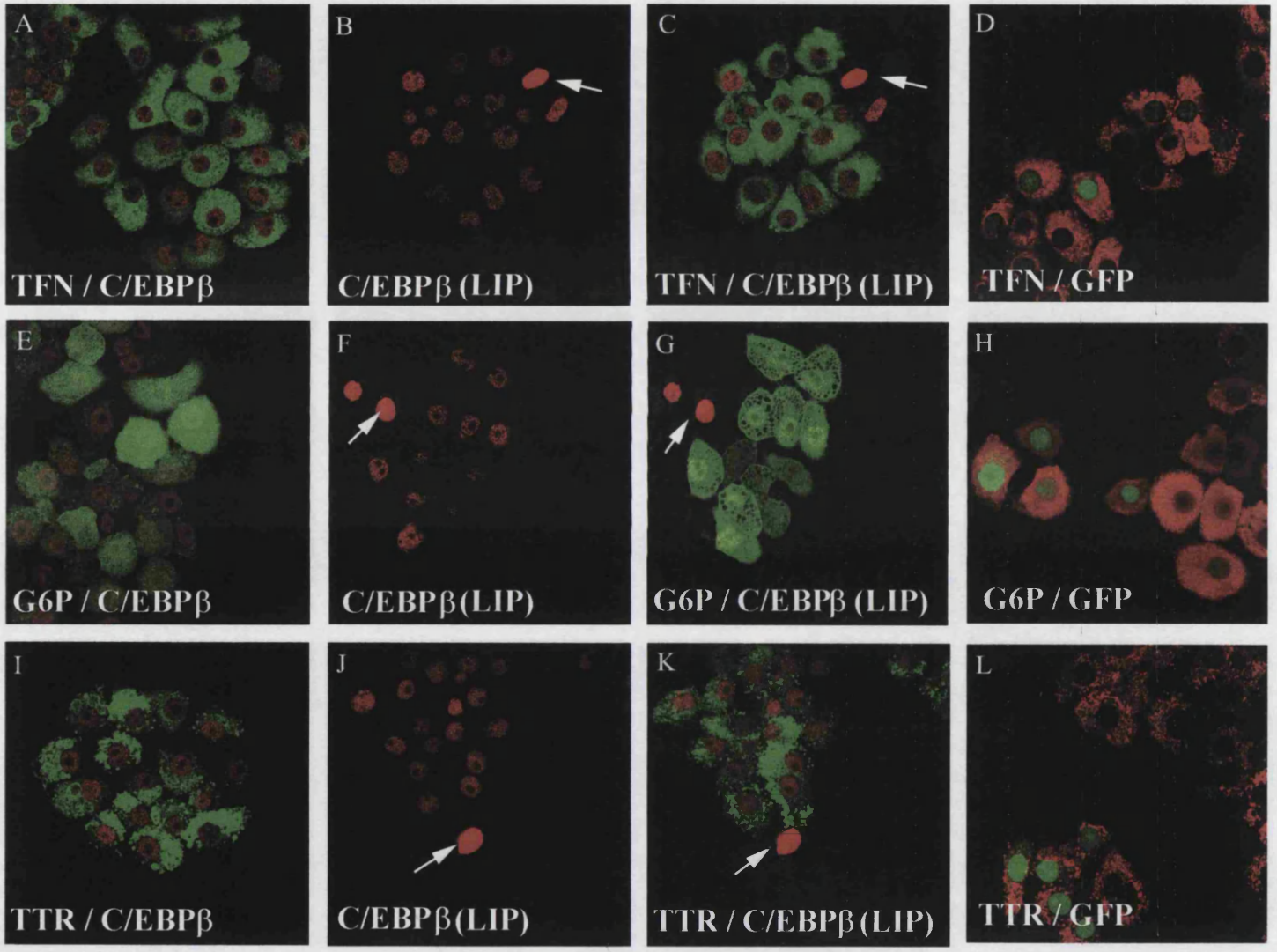

A
TFN / C/EBPβ
B
C/EBPβ (LIP)
C
TFN / C/EBPβ (LIP)
D
TFN / GFP
E
G6P / C/EBPβ
F
C/EBPβ (LIP)
G
G6P / C/EBPβ (LIP)
H
G6P / GFP
I
TTR / C/EBPβ
J
C/EBPβ (LIP)
K
TTR / C/EBPβ (LIP)
L
TTR / GFP
Dex+OSM 4d
LIP transfected
Dex+OSM 4d
GFP transfected
Dex+OSM 4d

Fig 4.7 Inhibition of transdifferentiation by LIP. LIP can be detected by immunohistochemistry because the C/EBPβ antibody is raised to the C-terminus. LIP transfected cells are much more intensely stained than those in which endogenous C/EBPβ has been induced.

Cells transfected with CMV-LIP (A-C, E-G, I-K) and after 24hrs treated with 1 μM Dex and 10 ng/ml OSM for 4 days. The cells were then immunostained for C/EBPβ (red) and TFN (A, C), G6Pase (E, G) or TTR (I, K) (green) respectively.

Cells transfected with CMV-nucGFP (D, H, L) and after 24hrs treated with 1 μM Dex and 10 ng/ml OSM for 4 days. The cells were then immunostained.for GFP (D, H, L) (green) and TFN (D), G6Pase (H) or TTR (L) (red). (B), (F) and (J) LIP transfected cells stain intensely (arrowed) whereas less intense cells show induced C/EBPβ

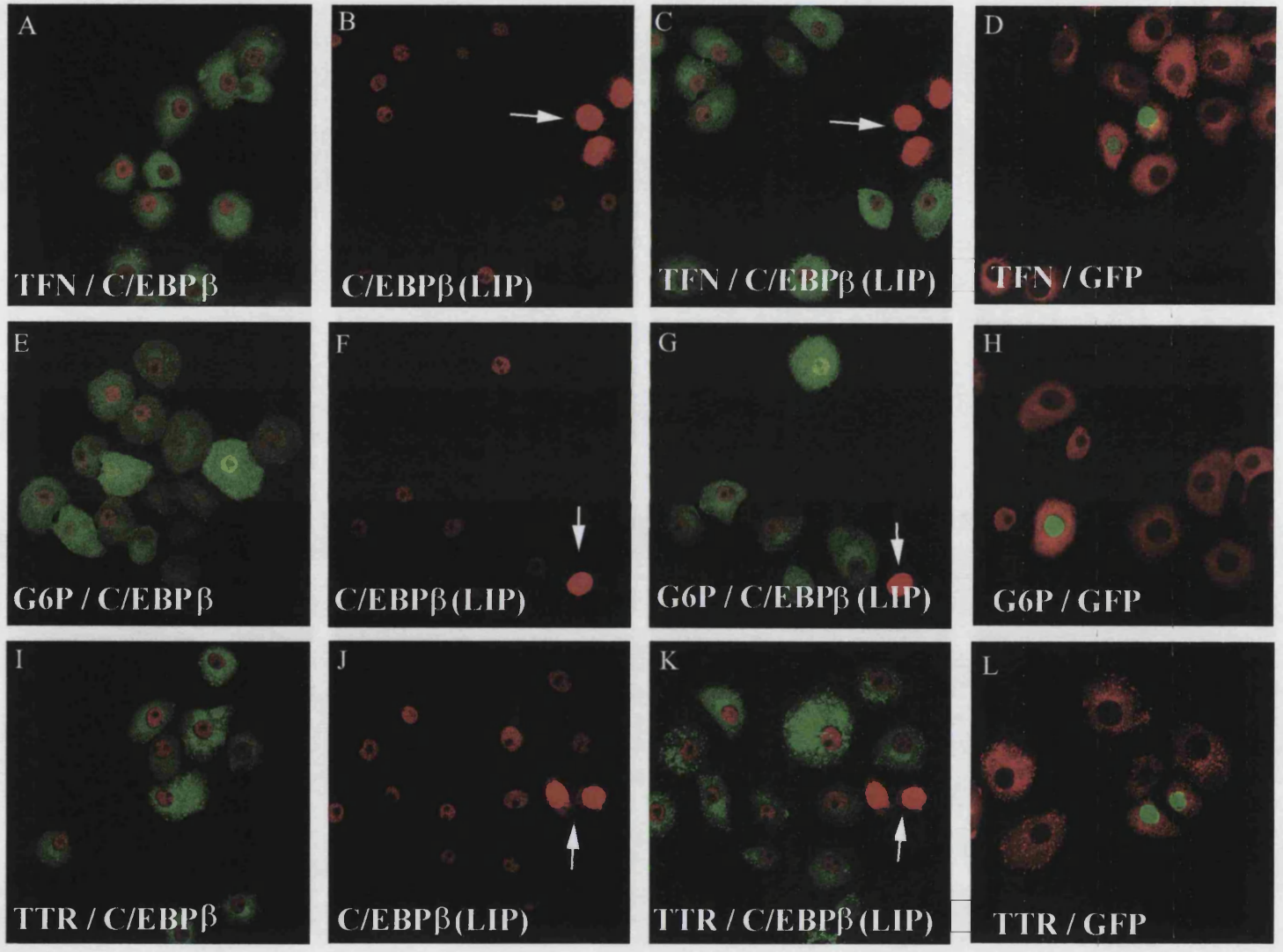

A
TFN / C/EBPβ
B
C/EBPβ(LIP)
C
TFN / C/EBPβ (LIP)
D
TFN / GFP
E
G6P / C/EBPβ
F
C/EBPβ(LIP)
G
G6P / C/EBPβ (LIP)
H
G6P / GFP
I
TTR / C/EBPβ
J
C/EBPβ(LIP)
K
TTR / C/EBPβ (LIP)
L
TTR / GFP
Control
LIP transfected
GFP transfected

Fig 4.8 C/EBPβ is essential for maintenance of the transdifferentiation.

AR42J-B13 cells were treated for 8-10 weeks with 1 μM Dex, then cultured with 1 μM Dex and 10 ng/ml OSM for 5 days prior to transfection with CMV-LIP (A-C, E-G, I-K) or CMV-nucGFP (D, H, L), followed by exposure to Dex+OSM for a further 72 hrs and then immunostained for C/EBPβ (red) and TFN (A, C), G6Pase (E, G) or TTR (I, K) (green) respectively, or GFP (D, H, L) (green) and TFN (D) , G6Pase (H) or TTR (L) (red). (B), (F) and (J) LIP transfected cells stain intensely (arrowed) whereas less intense cells show induced C/EBPβ

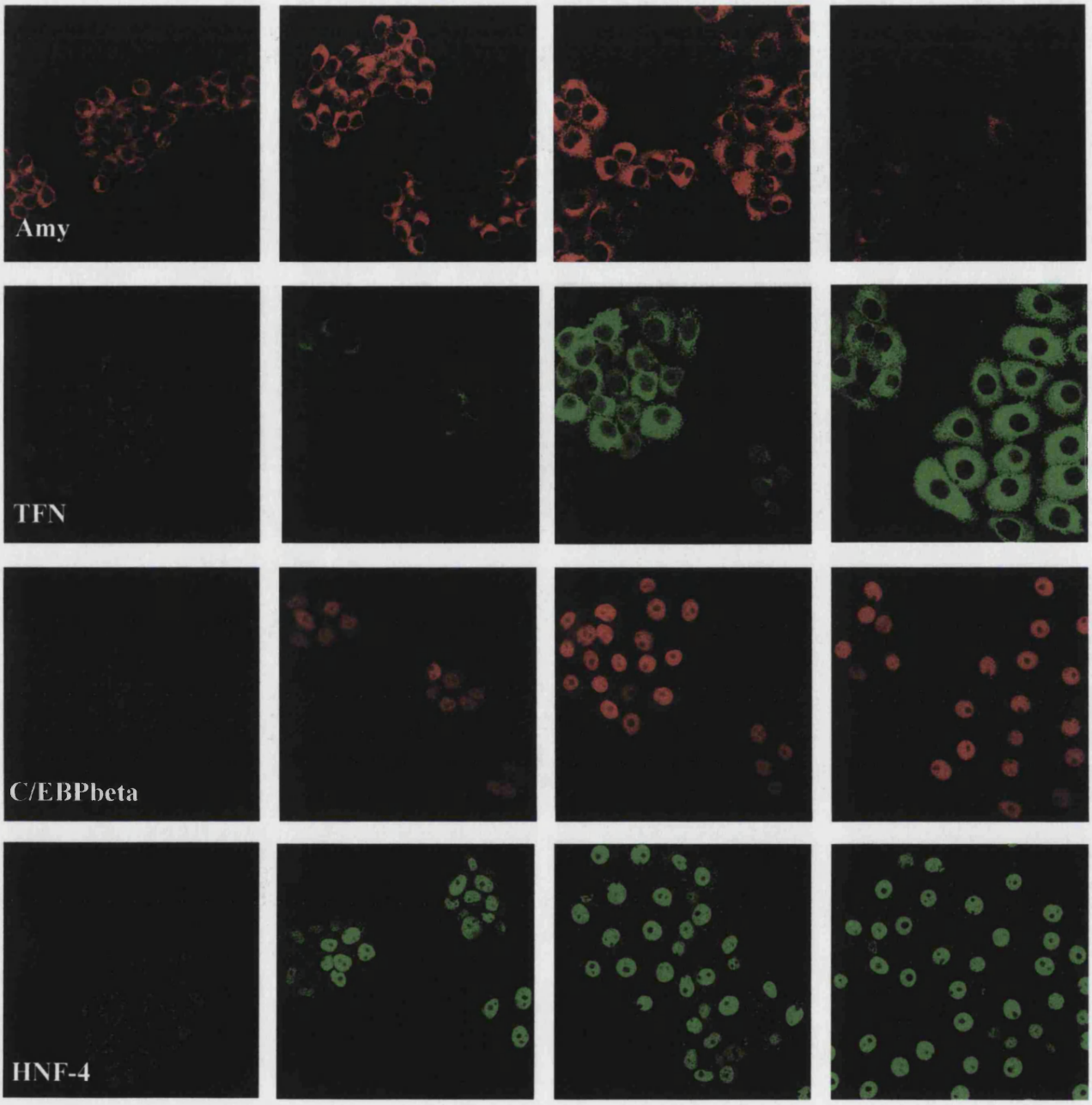
4.5 Regulation of epithelial morphogenesis in transdifferentiation

In epithelia, adhesive interactions participate in specific histogenetic events and also instate a morphological polarization that is linked to the vectorial functioning of the epithelium (Stamatoglou and Hughes, 1994). The polarised configuration of hepatocytes in the liver is crucial in maintaining liver function. When isolated hepatocytes are cultured in vitro, they lose the characteristic pattern of gene expression and their polarized configuration. Addition of hormones to the culture or growth of hepatocytes inside collagenous matrices or matrigel (Engelbreth-holm-swarm tumour-derived gel matrix) can counteract the polarized configuration and differentiation to varying degree (Jefferson et al., 1984; Isom et al., 1985; Duun et al., 1992; Ben-Ze'ev et al., 1988)

In previous chapters, we showed that following treatment with dexamethasone the pancreatic cells convert to cells expressing several liver-specific markers which are all expressed in differentiated hepatocytes. Associated with the transdifferentiation we also observed changes in cellular morphology. Following removal of Dex from the medium, we found some cells lost their epithelia-like morphology accompanied by loss of liver maker expression. The observations indicate that the mechanism regulating the transdifferentiation of pancreas to liver may also regulate hepatic morphogenesis and liver differentiation. As a result, this transdifferentiation model might provide us an opportunity to investigate the mechanisms involved.

We firstly analysed the expression of different cytoskeleton or cell adhesion markers which were known to be involved in formation of hepatic morphology and polarity including F-actin, Pan-cytokeratin, E-cadherin, and β-catenin. These results show that an increase of filopodia-like structures from day 2 is accompanied by the down-regulation of pancreatic amylase (Fig 4.9). Cells then become flattened,

enlarged, express hepatic transferrin and form epithelial tight junctions which are associated with the expression of E-cadherin, and submembrane localisiation of β-catenin. Rearrangement of cytokeratin proteins is also observed during the transdifferentiation.


Amy
TFN
C/EBPbeta
HNF-4
Control
Dex + OSM 2d
Dex + OSM 3d
Dex + OSM 5d

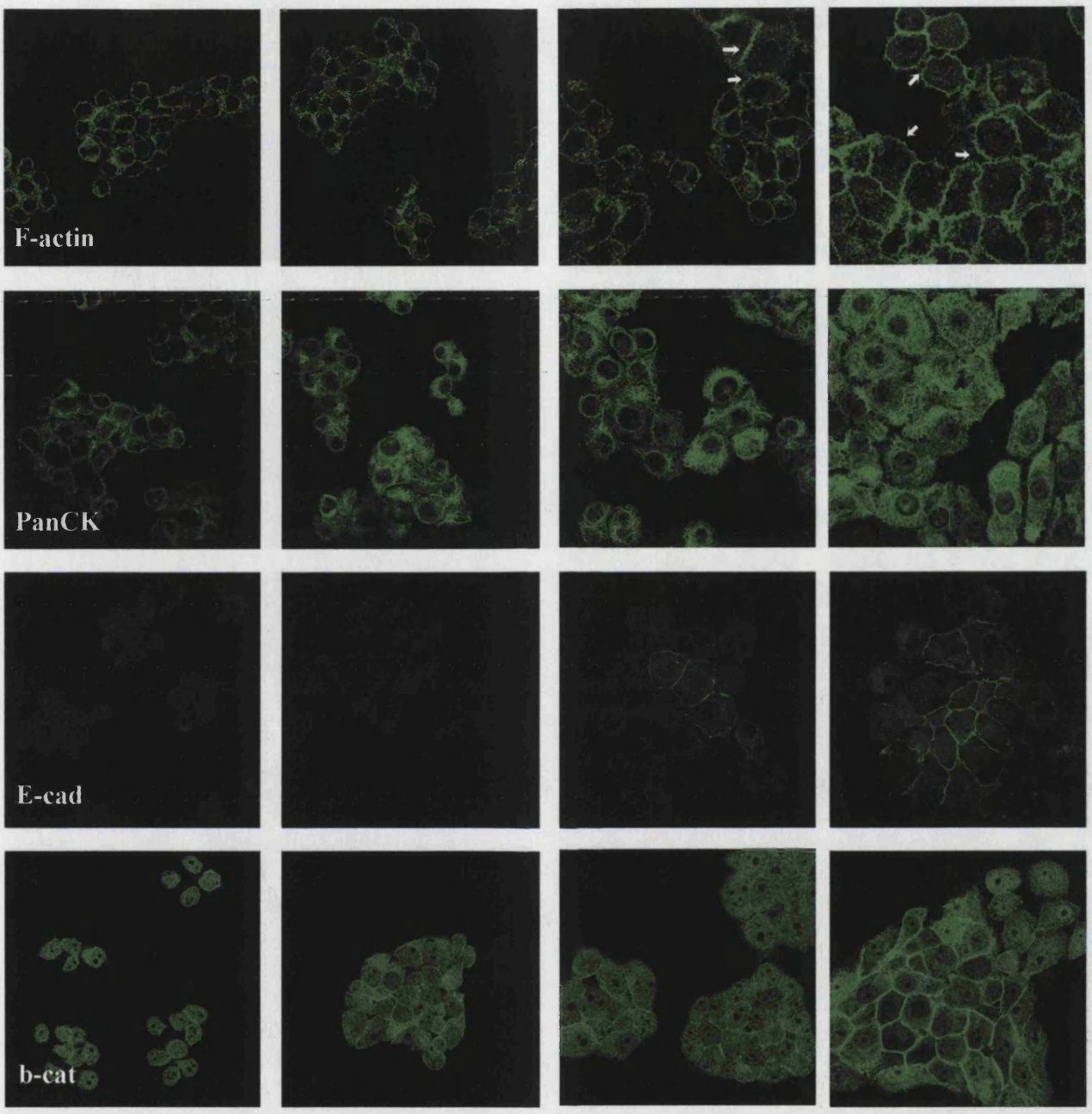

F-actin
PanCK
E-cad
b-cat
Control
Dex + OSM 2d
Dex + OSM 3d
Dex + OSM 5d

Fig 4.9 Time course experiments showing that the hepatic transdifferentiation is associate with down-regulation of pancreatic amylase and expression of E-cadherin, and submembrane localisiation of β-catenin. Rearrangement of Pan-cytokeratins has also been observed during the transdifferentiation. AR42J-B13 cells were treated for 2, 3, 5 days with 1 μM Dex and 10ng/ml OSM, and then fixed and immunostained for amylase (Amy), transferrin (TFN), C/EBPβ, HNF-4α, phalloidin-FITC (F-actin), Pan-Cytokeratin (PanCK), E-cadherin (E-cad) and β-catenin (b-cat). Arrows indicate the formation of filopodia-like structure.

Previously, we have shown that transfection of LIP (liver inhibitory protein), the dominant negative form of C/EBPβ is sufficient to inhibit transdifferentiation. Here, we also show LIP can also down-regulate the formation of filopodia-like structures, and the expression of cytokeratins and E-cadherin (Fig 4.10). In order to determine whether the morphogenesis is essential for the transdifferentiation, I decide to add cytochalasin D, an inhibitor of actin polymerisation, to the cultures to inhibit the morphogenesis. Here, we found addition of cytochalasin D at day 2 can prevent epithelial morphogenesis and onset of liver gene expression (Fig 4.11).

In order to determine whether the inhibitory effect was caused by the toxicity of the cytochalasin D, I remove cytoclasin D from day 3, and then cultured the cells with or without 1 μM Dex and 10 ng/ml OSM. The results showed that the cells can still transdifferentiate into hepatocytes (Fig 4.12) indicating cytochalasin D treatment, which prevents epithelial morphogenesis and hepatic transdifferentiation is not the effect of toxicity. It actually suggests epithelial morphogenesis may play an important role in the hepatic transdifferentiation of pancreas.

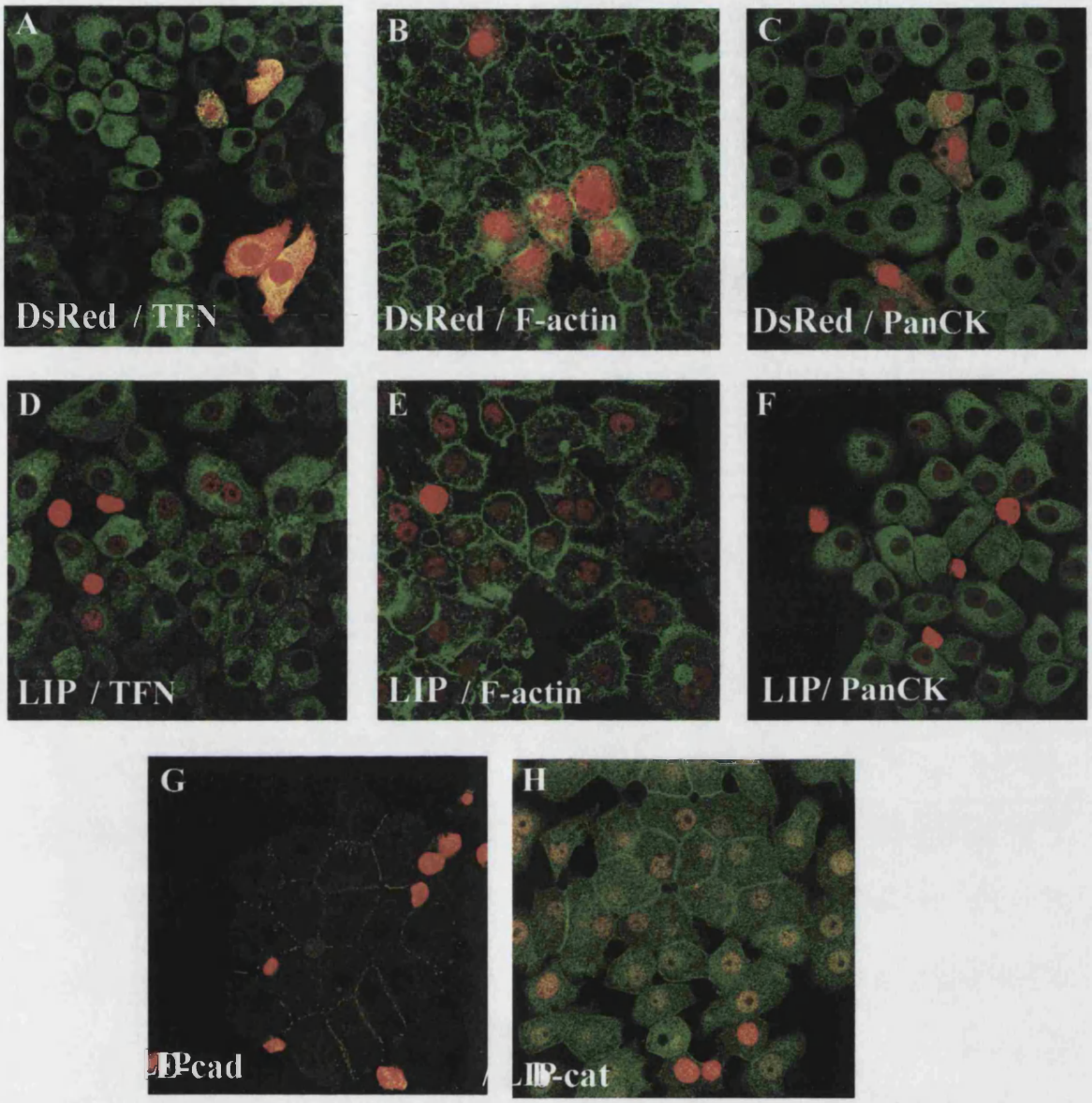

A
DsRed / TFN
B
DsRed / F-actin
C
DsRed / PanCK
D
LIP / TFN
E
LIP / F-actin
F
LIP/ PanCK
G
H

Fig 4.10 Transfection of LIP can not only inhibit the expression of transferrin, but it can also down-regulate the formation of filopodia-like structures, and the expression of cytokeratins and E-cadherin. AR42J-B13 cells were transfected with CMV-DsRed (A-C) or LIP (D-H), and then treated with 1 μM Dex and 10 ng/ml OSM for 4 days and then immunostained for: DsRed (Red, A-C), C/EBPβ (Red, D-F), transferrin (Green, A&D), phalloidin-FITC (Green, B&E), Pan-Cytokeratin (Green C&F), E-cadherin (Green, G) and β-catenin (Green, H)

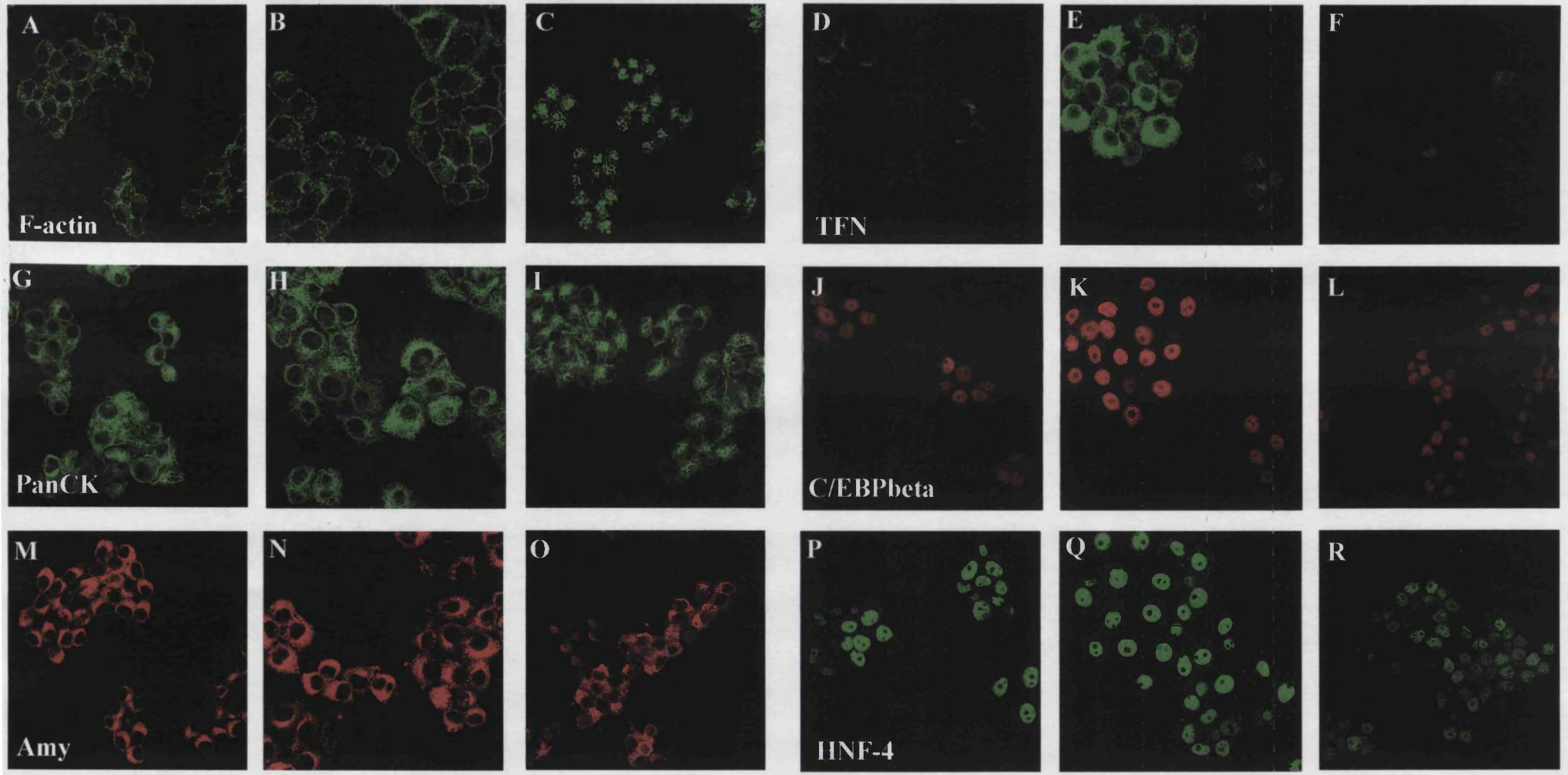

A
F-actin
B
C
D
TFN
E
F
G
PanCK
H
I
J
C/EBPbeta
K
L
M
Amy
N
O
P
HNF-4
Q
R
Dex+OSM 2d
Dex+OSM 3d
Dex+OSM 3d
+ 2.5 uM cyto D
Dex+OSM 2d
Dex+OSM 3d
Dex+OSM 3d
+ 2.5 uM cyto D

Fig 4.11 Addition of cytochalasin D can inhibit actin polymerisation, morphological changes and expression of transferrin. AR42J-B13 cells were treated with 1 μM Dex and 10 ng/ml OSM for 2-3 days. Addition of 2.5 μM cytochalasin D to some cultures at day 2 (C, I, O, F, L R), and then immunostained for phalloidin-FITC (A-C), transferrin (D-F), Pan-Cytokeratin (G-I), C/EBPβ (J-L), amylase (M-O), and HNF-4 (P-R)

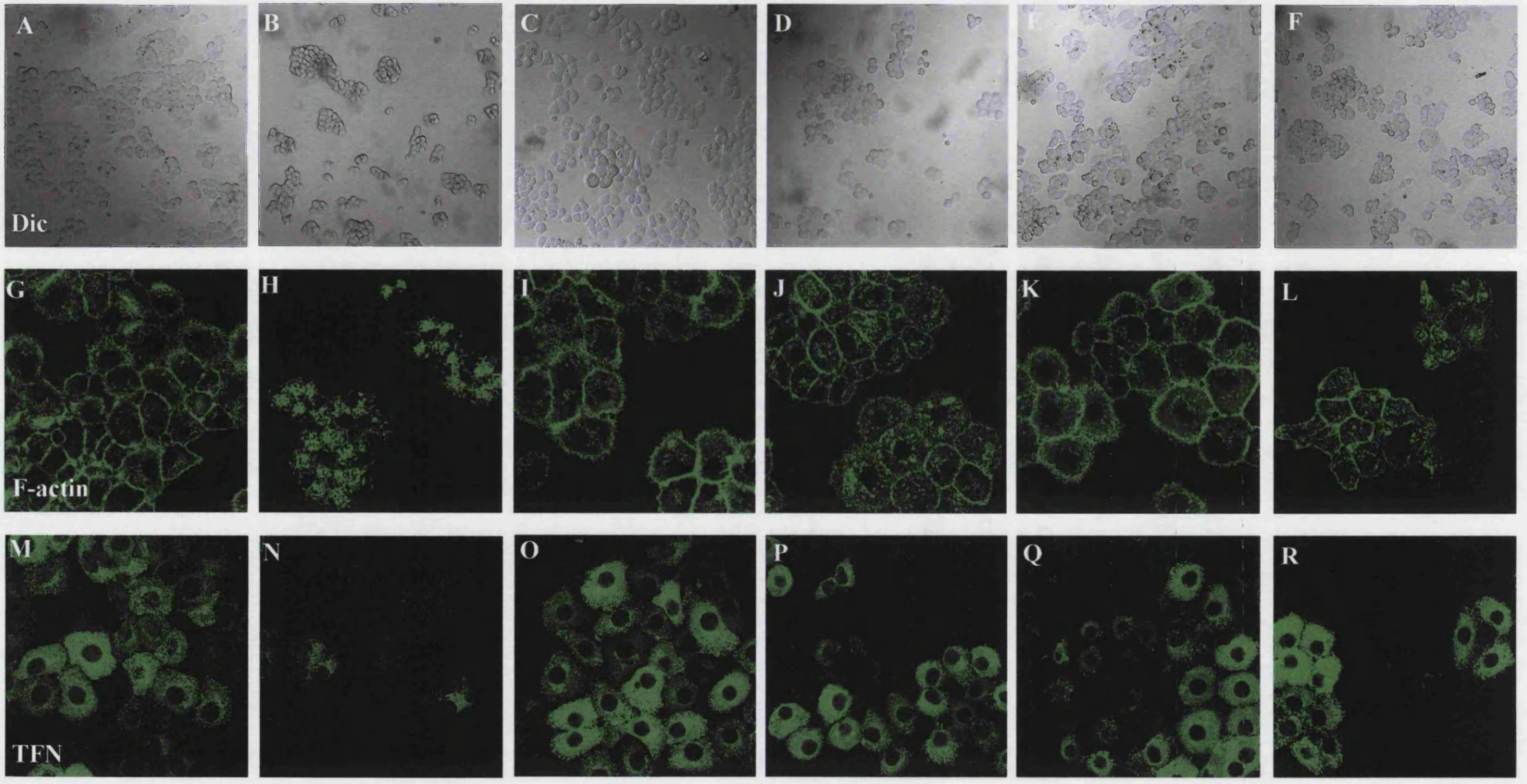

A
B
C
D
E
F
Dic
G
H
I
J
K
L
F-actin
M
N
O
P
Q
R
TFN
Dex+OSM 3d
Dex+OSM 3d
2.5 uM cD d2-d3
Dex+OSM 5d
Dex+OSM 5d
2.5 uM cD d2-d3
Dex+OSM 3d2r
Dex+OSM 3d2r
2.5 uM cD d2-d3

Fig 4.12 Removal of cytochalasin D from day 3 culture, cells can continue the transdifferentiation and morphogenesis programme. AR42J-B13 cells were treated with 1 μM Dex and 10 ng/ml OSM for 2 days, and then 2.5 μM cytochalasin D were added to some cultures at day 2 (B, H, N, D, J, P, F, L, R). Cytochalasin D was then removed from day 3, and cultured with (C-P) or without Dex+OSM (E-R). Cells were fixed at day 3 or day 5 and immunostained for phalloidin-FITC (G-L) and transferrin (M-R).

4.6 Discussion

In the experiments described in this chapter, we have determined the molecular events associated with the transformation of AR42J-B13 cells to hepatocytes, are induction of C/EBPβ followed by the nuclear translocation of HNF-4α and the activation of differentiated hepatic products. Transfection of C/EBPβ into the cells can itself provoke the transdifferentiation while LIP (liver inhibitory protein), the dominant-negative form of C/EBPβ can inhibit the process, suggesting that C/EBPβ is a key component distinguishing between the liver and pancreatic programs of differentiation.

The results mimic the hepatic metaplasia of pancreas observed in animal experiments. In the copper deprivation regime the exocrine pancreas mainly degenerates and becomes replaced by fibrous tissue. When the animals are re-fed on a normal diet there is a rapid accumulation of fat in this interstitial tissue. The exocrine acini then regenerate over several weeks from small foci, and are accompanied by the growth of foci of hepatocytes. So in the course of this treatment, individual surviving exocrine cells would be exposed to conditions very different to those experienced in the normal intact pancreas. Since C/EBPβ is known to be involved in adipogensis (Tanaka et al., 1997; Darlington et al., 1998), the accumulation of fat in the interstitial tissue could be caused by the generation of C/EBPβ. C/EBPα, β and δ mRNAs have been shown to increase in copper-deprived pancreas, at about 4-5 weeks, just before the appearance of visible hepatocytes. It is therefore likely that C/EBPβ is involved in the process of metaplasia *in vivo*, but it is not determined whether the C/EBPs are directly involved in the process.

Evidence from mouse knockouts have so far not shown a major function in normal liver development for C/EBPs, but C/EBPβ has been shown to act as a key gene for liver regeneration (Diehl 1998). Actually, the knockouts of *C/EBPα* and *C/EBPβ* do have slight liver defects (Wang et al., 1995; Screpanti et al., 1995; Liu et al., 1998). We suspect that simultaneous knockouts of all C/EBP type genes may cause a major liver defect and it is significant that the deletion of the C/EBP homologue in *Drosophila* is embryonic lethal (Rørth and Montell, 1992). Additionally, in vivo footprinting shows that in embryonic hepatocytes, C/EBPβ is actually bound to the albumin gene enhancer (Bossard et al., 1997). This result indicates a role of C/EBPβ in fetal liver development.

The hepatocyte nuclear factor 4 (HNF-4) is a member of the nuclear receptor superfamily (Sladek et al., 1990; Mangelsdorf and Evans, 1995). These factors normally exist in the cytoplasm, and the ligand-induced translocation to the nucleus is an important step in regulating their target gene transcription. HNF-4 is abundant in adult as well as the fetal liver and intestine, but is present at a lower level in islets of Langerhans and is scarcely detectable in the exocrine pancreas (Miquerol et al., 1994). HNF-4 is known to play a critical role in regulating the function of normal pancreatic β-cell, because a mutation in this factor causes maturity onset diabetes of the young type 1 (MODY1) (Yamagata et al., 1996b).

The importance of HNF-4 in liver differentiation comes from oval cell regeneration. If rats are treated with aminoacetyl-fluorene to inhibit hepatocyte growth, then subjected to partial hepatectomy, liver regeneration proceeds from oval cells associated with the bile ducts (Alison et al., 1998). During this process, the expression of HNF-4 is first observed when the oval cells differentiate morphologically and functionally into hepatocytes (Nagy et al., 1994). These results all imply that the

activity of HNF-4 plays a critical role in regulating liver differentiation, both in development and in regeneration.

Weiss and coworkers found HNF-4 expression overcomes repression of the hepatic phenotype in dedifferentiated hepatoma cells, and glucocorticoid enhances this effect (Spath and Weiss, 1997; 1998). They also found a similar phenomenon as we describe here in that the differentiation is associated with growth arrest. Furthermore, Weiss and colleagues also suggest that HNF-4 seems to integrate the genetic programs of liver-specific gene expression and epithelial morphogenesis (Spath and Weiss 1998). They found HNF-4 is sufficient to provoke reexpression of a set of hepatocyte marker genes and also to the reestablishment of differentiated epithelial cell morphology and simple epithelial polarity in dedifferentiated rat hepatoma H5 cells. Expression of HNF-4 results in reexpression of cytokeratin proteins and partial reestablishment of E-cadherin production. Only the HNF-4 transfectants are competent to respond to the synthetic glucocorticoid dexamethasone, which induces the second step of morphogenesis, including formation of the junctional complex and expression of a polarized cell phenotype.

Recently Fuchs and coworkers found Calcium stimulates formation of filopodia in keratinocytes, which penetrate and embed into neighboring cells. E-cadherin complexes cluster at filopodia tips, generating a two-rowed zipper of embedded puncta (Vasioukhin et al., 2000). In this chapter I demonstrate that enhancement of actin polymerisation and formation of filopodia-like structures during the early phase, which may promote cells to make initial contact with neighbours. Cells then become flattened, start to express hepatic transferrin and form epithelial tight junctions which are associated with expression of E-cadherin, and submembrane localisiation of β-catenin. Rearrangement of cytokeratin proteins has been shown to

occur during the transdifferentiation. Cytochalasin D can prevent epithelial morphogenesis and liver gene expression suggesting a possible role for microfilaments in the transdifferentiation process. These results suggest epithelial morphogenesis may play an important role in the hepatic transdifferentiation of pancreas, but it remains to be determined whether HNF-4 is essential.

In the developing mouse, HNF-4 is normally expressed in the early visceral endoderm, an extraembryonic tissue which shares many biochemical features with the liver. Disruption of the HNF-4 genes in visceral endoderm blocks the expression of α-fetoprotein, apolipoprotein, transthyretin and transferrin (Duncan et al., 1997). The knockout mouse dies early because of failure of the visceral endoderm. Recent studies performed by Duncan and his colleagues showed that HNF-4 -/- fetal liver failed to express a large array of genes, which are essential for hepatic function (Li et al., 2000). The results suggest an essential role of HNF-4 in liver differentiation.

We therefore propose that the transformation from pancreas to liver arises from a molecular pathway in which Dex activates the glucocorticoid receptor; this activates transcription of C/EBPβ, and translocation of HNF-4α to the nucleus and activation of target genes. The target genes comprise α_1-antitrypsin, transferrin, transthyretin and glucose-6-phosphatase, and other markers characteristic of early liver differentiation.

CHAPTER 5

FORMATION OF

HEPATOCYTES IN PANCREATIC BUD CULTURE

5.1 Introduction

The mature pancreas has morphologically and functionally distinct endocrine and exocrine components. The exocrine portion, including acinar and ductal cells, comprises 95%–99% of the pancreas and produces digestive enzymes that promote nutrient digestion and absorption in the gut. Mature endocrine cells are located in the islets of Langerhans and aggregated scattered throughout the exocrine pancreas. Islets contain four principal endocrine cell types, defined by the hormones they secrete. These include insulin-producing β-cells, glucagon-producing α-cells, somatostatin-producing δ-cells, and pancreatic polypeptide–producing PP cells. All pancreatic cell types (endocrine, exocrine, and ductal) are derived from the same endodermal dorsal and ventral primordium, which grow together to form the definitive pancreas. (Slack 1995; Edlund 1999; Kim and Hebrok, 2001)

Early pancreatic development has been particularly well studied in mice and chickens, and important similarities and differences in pancreatic morphogenesis in these species have been previously described (Kim et al. 1997b). Here, I will briefly describe the development of the pancreas in mice. During embryogenesis, the pancreas develops from distinct dorsal and ventral anlagen. Before specification toward a dorsal pancreatic fate, the midline endoderm in the posterior foregut is a single layer of epithelial cells that contacts the notochord, an axial mesoderm-derived structure (Kim and Hebrok, 2001). Laterally, endoderm fated to form ventral pancreas is adjacent to both splanchnic mesoderm and aortic endothelial cells but is not in direct contact with the notochord. The notochord and dorsal pancreatic endoderms remain in contact until about the 13-somite stage in mice (8.5d postcoitum (dpc)), when midline fusion of the paired dorsal aortas occurs. The first indication of morphogenesis occurs at 22–25 somites in mice (9.5 dpc), when the dorsal mesenchyme condenses and

underlying endoderm evaginates, forming a recognizable dorsal pancreatic bud; the ventral bud appears later at 30 somites (10.5 dpc). Stimulated by mesenchymal signals, pancreatic epithelial cells proliferate and branch (Slack 1995; Kim and Hebrok, 2001).

In the developing pancreatic buds, the endocrine cells start to differentiate before the exocrine cells, and co-expression of different hormones by the same cell is often observed at early stages. Although pancreatic endocrine cells produce many gene products also characteristic of neurons, evidence from *in vitro* cultures and from chick grafts shows that they are of endoderm and not of neural crest origin. Observational and experimental recombination studies suggest strongly that both endocrine and exocrine cells arise from the same endodermal rudiment (Slack 1995; Percival and Slack, 1999).

Assuming that the principles of development as established by work on early embryos holds also for organ development, there must be a region within the endoderm committed to form the pancreas at some stage before the appearance of the first terminal differentiation products. This region presumably consists of a set of cells committed by the expression of a particular combination of transcription factors. Obviously, it would be good to know the identity of the genes involved in the specification of the pancreas. Golosow and Grobstein were pioneers in the field of pancreas developmental research, as were Wessells and Cohen, who already in the 1960s performed classic embryological experiments describing the morphogenesis of the pancreas and the epithelio-mesenchymal interactions that are instrumental for proper pancreas development. Recent findings suggest that follistatin and fibroblast growth factors represent some of these key mesenchymal factors that actively promote at least pancreatic exocrine development (Review in Edlund 2001). The early steps that control the commitment of a region of localized gut epithelium to a pancreatic fate

and the mechanism underlying the specification of the different pancreatic cell types are, however, not well understood. One approach to determining the mechanism is to investigate the occurrence of heterotopic pancreas in the embryo, and also the hepatic metaplasias that can be displayed by a regenerating pancreas in the adult. Both suggest that only a few gene products distinguish the pancreatic cell state from that of the surrounding tissues of duodenum, gall bladder and liver (Slack 1995).

In previous chapters, I have shown that dexamethasone can induce pancreatic AR42J-B13 cells to transdifferentiate into hepatocytes. Like all tissue culture cells, the AR42J-B13 cells are undoubtedly somewhat altered since their ancestors were isolated 20 years ago. In order to establish that the results obtained were not just a tissue culture artifact but were relevant to the situation *in vivo*, in this chapter we studied the effects of dexamethasone on pancreatic buds isolated from mouse embryos.

5.2 Development of pancreatic buds *in vitro*

In mice, soon after specification of the pancreatic anlage in posterior foregut endoderm at 8.5 dpc, somatostatin mRNA becomes detectable, marking the beginning of endocrine cell differentiation (Herrera et al. 1991; Gittes and Rutter 1992). Immunolocalisation demonstrates the appearance of scattered cells expressing insulin or glucagon from 9.5 dpc. Development of exocrine cells into acini and ducts is recognizable by 14.5 dpc; at this stage, endocrine cells are found embedded as individual cells in ducts or in small cell clusters distinct from ducts. The stereotyped architecture of islets with central insulin-expressing β-cells surrounded by α-cells is detected late in gestation from 18 dpc (Slack 1995; Edlund 1999; Kim and Hebrok, 2001).

Golosow and Grobstein (1962) found that an 11.5d mouse pancreatic bud would develop very well *in vitro*, but that the isolated epithelium would not grow or differentiate in the absence of mesenchyme (Reviewed in Kim and Hebrok, 2001). Previously our lab have devised a new culture system for *in vitro* culture of pancreatic buds from mouse embryos which enables the organ to grow as a flat branched structure suitable for wholemount immunostaining (Percival and Slack 1999). This system has been used to analyze pancreatic development (Percival and Slack 1999; Horb and Slack 2000).

The dorsal buds were isolated from E11.5 mouse embryos. The buds adhere to the fibronectin substrate within a few hours and gradually flatten out over the first 1–2 days. Mesenchymal cells spread rapidly out of the explant to form a monolayer of cells surrounding the clump in the centre. On the second or third day, branches begin to appear in the epithelium. We can find the appearance of scattered cells expressing insulin or glucagon, which became quite numerous from 13.5 days onward (Fig 5.1A). Over the next 3 days, the epithelium becomes an extended branched structure radiating from the original centre, and development of exocrine cells can be recognized (Fig 5.1B). Insulin cells are very few and faintly stained after 1 and 2 days of culture, but become more numerous and strongly stained thereafter. Clumps of endocrine cells resembling nascent islets can be seen scattered from day 6 (Fig 5.1C, D).

Our time course experiments showed that the cell differentiation in the cultures resembles quite closely what occurs *in vivo* although it occurred slightly later in time, the delay being about 1 day over a 4-day culture period. By contrast, as for all organ cultures of this type, the overall growth in size of the cultures is very much less than what is seen *in vivo.*

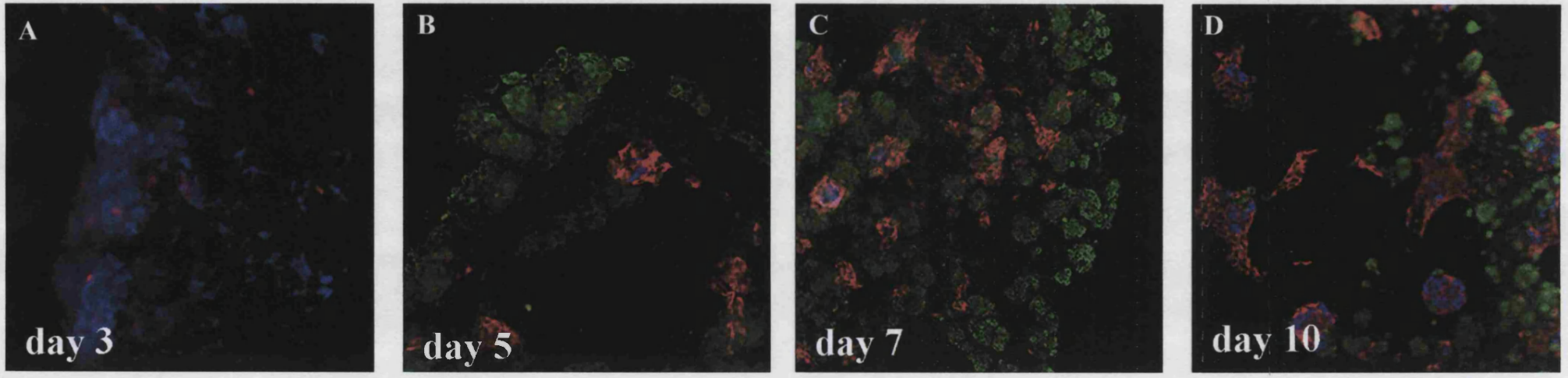

A
day 3
B
day 5
C
day 7
D
day 10

Fig 5.1 Development of pancreatic buds *ex vivo* Buds were cultured for 3, 5 7 and 10 days, fixed and stained for amylase (green), insulin (red) and glucagon (blue)

5.3 Formation of hepatocytes in pancreatic bud culture

In previous chapters, I have shown that dexamethasone can induce pancreatic AR42J-B13 cells to transdifferentiate into hepatocytes. Here, I would like to determine whether treatment with Dex can caused the hepatic transdifferentiation in pancreatic bud cultures. Dorsal pancreatic buds were isolated from 11.5d embryos and maintained in culture. After two days, Dex was added to the cultures. Firstly, we observe branching morphogenesis was reduced. After 5 days of Dex treatment, hepatocytes begin to appear within the buds as judged from expression of albumin, transferrin and α_1-antitrypsin (Fig.5.2). The result shows that the phenomenon of Dex-induced transdifferentiation is not restricted to the AR42J-B13 cells but applies equally to the normal developing pancreas.

The results obtained from AR42J-B13 cells suggest C/EBPβ is a key gene in the hepatic transdifferentiation, so I wanted to determine whether or not C/EBPβ is involved in the conversion in pancreatic bud culture. Firstly, I found that C/EBPβ was not normally expressed in the pancreatic buds, but became activated in many cells following Dex treatment, with concurrent reduction of amylase expression and activation of albumin expression in these cells (Fig.5.2E, Fig 5.3). The result again support the notion that C/EBPβ plays a major role in the transdifferentiation of pancreas to hepatocyte.

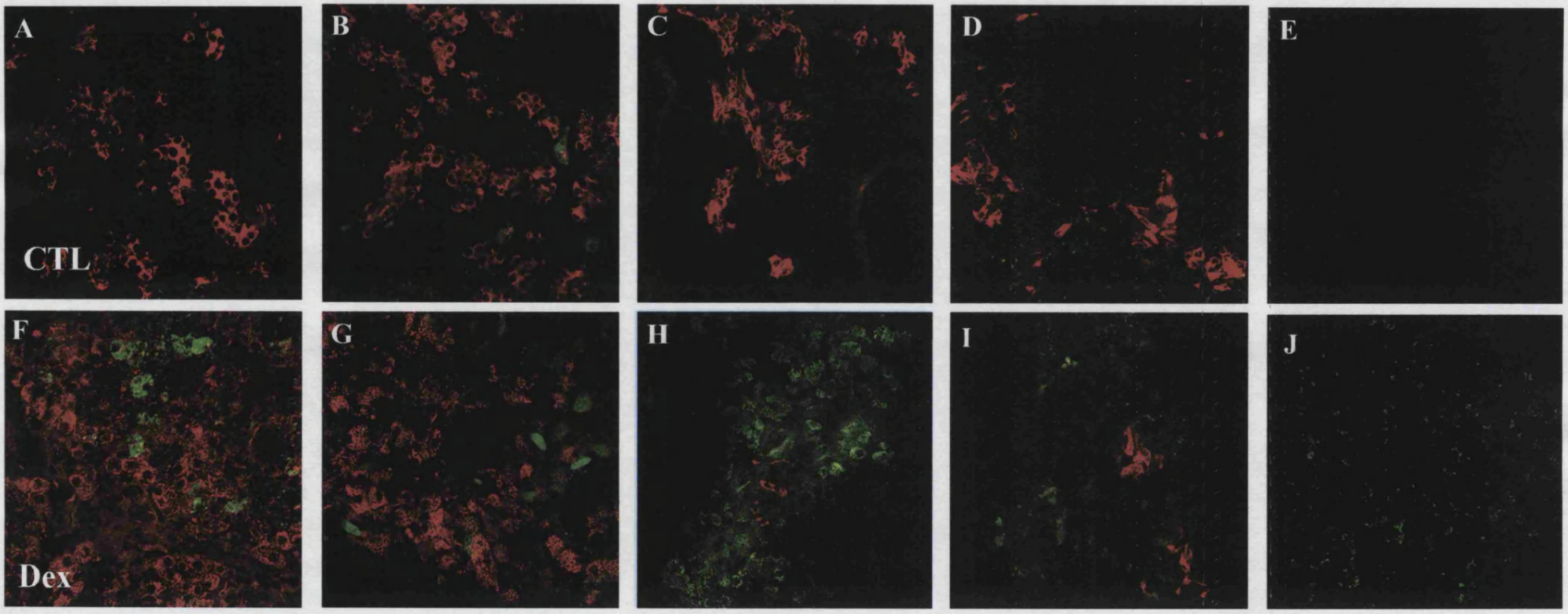

A
CTL
B
C
D
E
F
Dex
G
H
I
J
Alb / Amy
G6P / Amy
TFN / Ins
AAT / Ins
C/EBPbeta

Fig 5.2 Hepatic transformation in pancreatic buds from mouse embryos. Buds were cultured for 5 days without (A-E) or with 1 μM Dex (F-J), then immunostained for (A, F) albumin (green) / amylase (red). (B, G) glucose-6-phosphatase (green) / amylase (red). (C, H) transferrin (green) / insulin (red). (D, I) α_1-antitrypsin (green) / insulin (red). (E, J) C/EBPβ

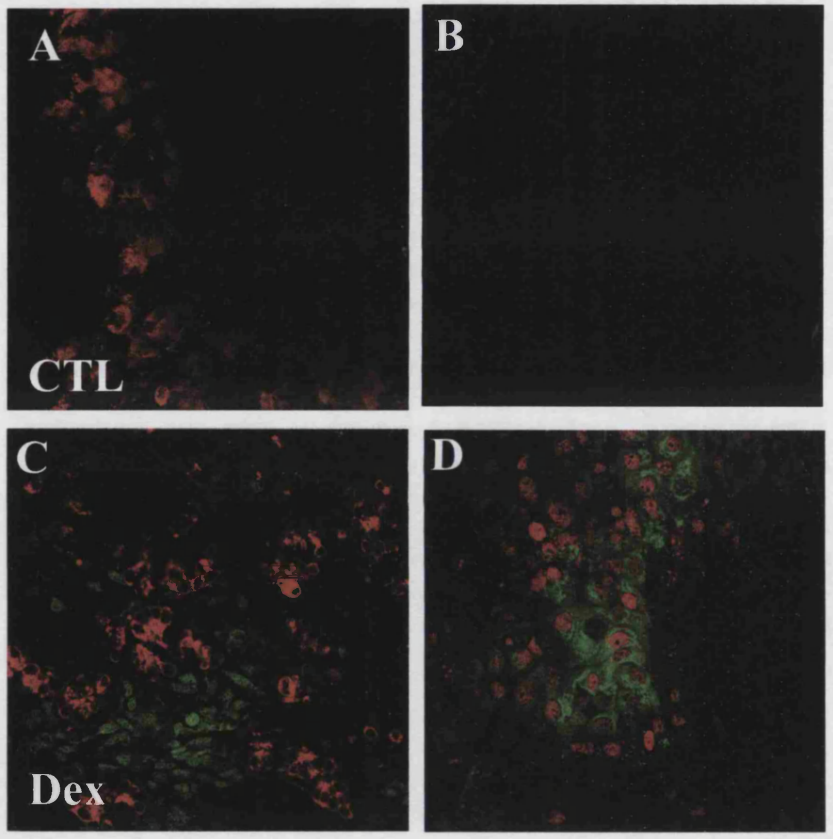

A
CTL
B
C
Dex
D
Amy/C/EBPβ
Alb/C/EBPβ

Fig 5.3 C/EBPβ expression during the hepatic differentiation in pancreatic buds from mouse embryos. Buds were cultured for 5 days without (A-B) or with (C-D) 1 μM Dex.

(A) Control, anti-C/EBPβ (green) and anti-amylase (red). C/EBPβ is not expressed.

(C) Dex treated, anti-C/EBPβ (green) and anti-amylase (red). C/EBPβ is expressed.

(B) Control, anti- C/EBPβ (red) and anti-albumin (green). Neither is expressed.

(D) Dex treated, anti-C/EBPβ (red) and anti-albumin (green). All cells expressing albumin also show C/EBPβ

Recently, Zaret and colleagues used tissue explant studies from 8.5 to 11.5 days gestation embryos to show that when the gut mesoderm is removed from the prospective intestinal endoderm, the endoderm activates the expression of liver-specific genes such as serum albumin, demonstrating the endoderm's pluripotence. But they also suggest at 13.5 d, the mesoderm gains a second inhibitory activity, resulting in the irreversible loss of plasticity (Bossard and Zaret 2000). In order to determine whether dexamethasone-induced hepatic transdifferentiation can occur after this stage, I set up bud culture from 13.5d embryos, and incubated for the further two days, then added Dex to the culture. After 5 days of Dex or Dex + OSM treatment, we still can find cells expressing transferrin (Fig 5.4), and there was no difference in expression of transferrin between Dex and Dex + OSM treatment. This result suggests that Dex treatment is sufficient to overcome the commitment of pancreatic development and redirect the cells to transdifferentiate into hepatocytes

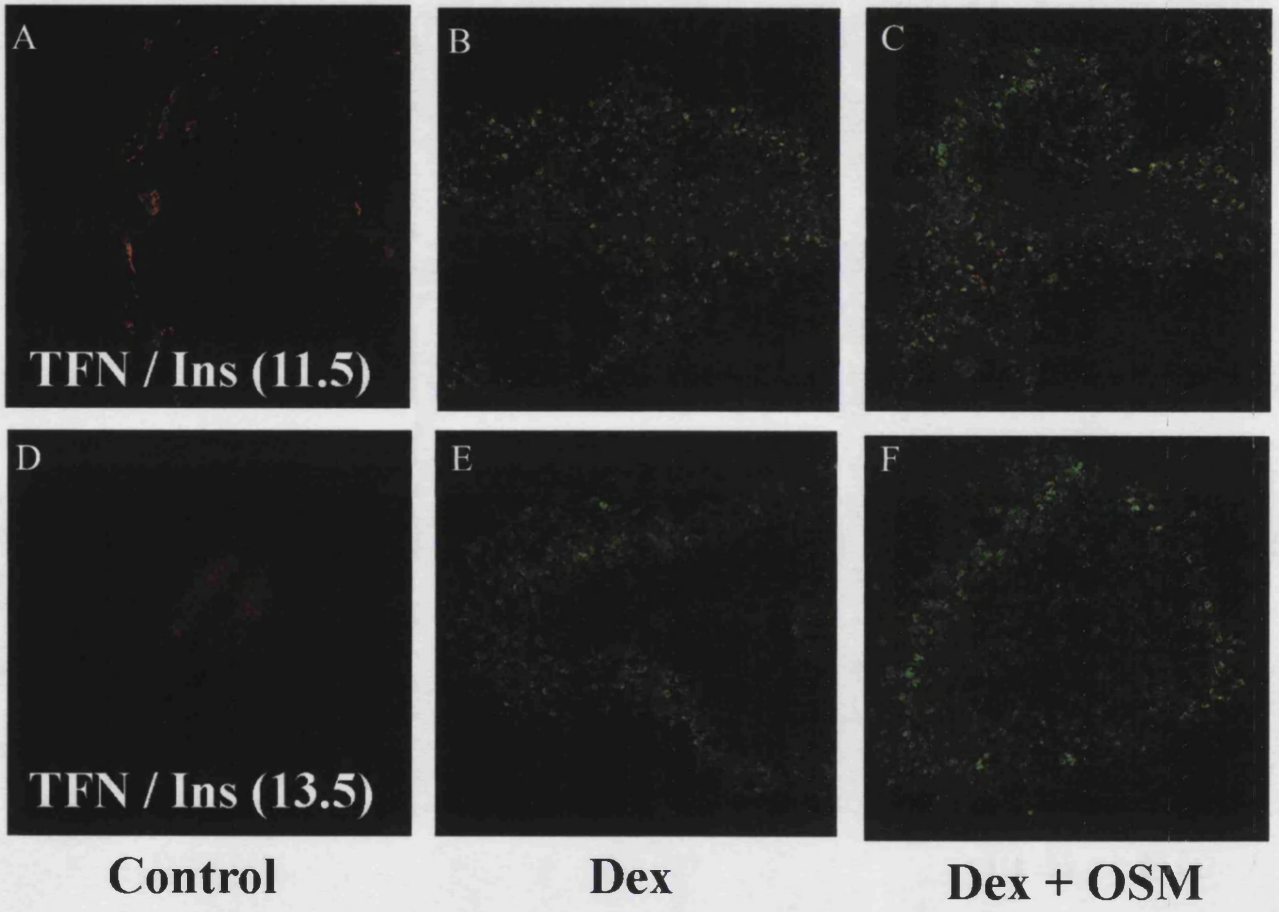

A
B
C
TFN / Ins (11.5)
D
E
F
TFN / Ins (13.5)
Control
Dex
Dex + OSM

Fig 5.4 Hepatic transformation in pancreatic buds from 13.5d mouse embryos. Buds were isolated from 11.5d (A-C) or 13.5d (D-F) mouse embryos. After two days, bud cultures were incubated without (A, D) or with 1 μM Dex (B, E) or 1 μM Dex + 10 ng/ml OSM (C, F) for 5 days. And then immunostained for anti-transferrin (green) and anti-insulin (red)

5.4 Origin of pancreatic hepatocytes

The accumulated evidence is consistent with the possibility that a unique cell gives rise to all pancreatic cell lineages although the existence of such a pancreatic "stem" cell remains debatable. In the AR42J-B13 cells, we set up a perdurance experiment to prove that some of hepatocytes arise from differentiated exocrine cells. Unfortunately, a similar lineage experiment is difficult to set up in organ culture because we are unable to transfect genes into the epithelium (data not shown). Interestingly, when Dex-treated cultures were stained for both albumin and amylase, it was found that albumin expression was often present in cells that are also positive for amylase. This was even more noticeable when the Dex was accompanied by oncostatin M (Fig.5.5). The finding is different from the result we have seen in AR42J-B13 cells- where the amylase is lost from all cells that have reached the stage of albumin expression, so in this respect the two systems differ. But the observation does support the conclusion from our GFP perdurance experiment on the AR42J-B13 cells that at least some of the hepatocytes arise by direct transdifferentiation from pancreatic exocrine cells. Again, it is not possible to say that hepatocytes do not also arise from stem cells or other types of progenitor cells.

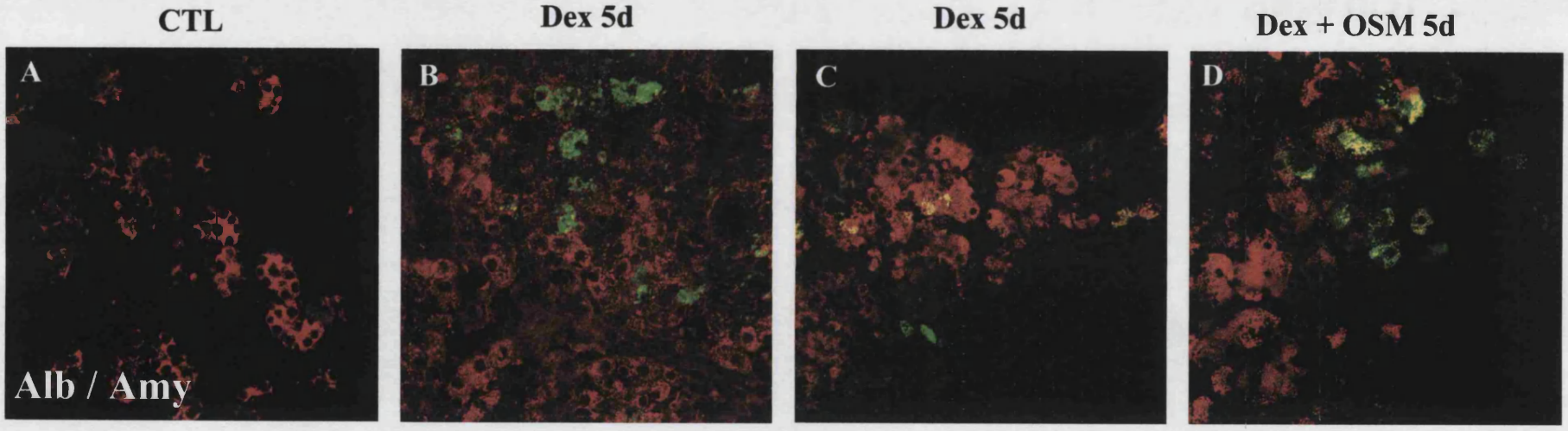
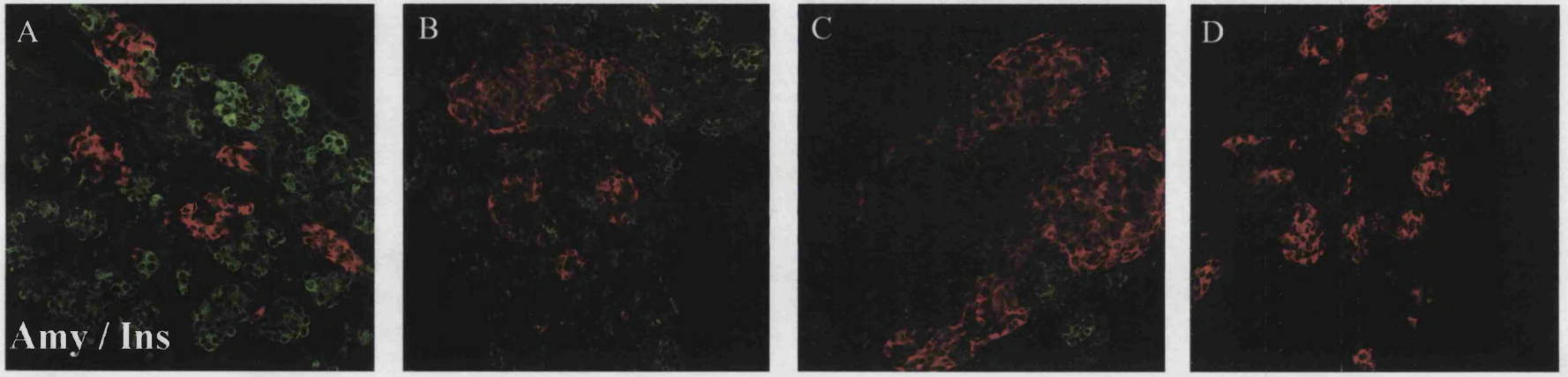

CTL
Dex 5d
Dex 5d
Dex + OSM 5d
A
B
C
D
Alb / Amy

Fig 5.5 Dex treatment caused co-expression of amylase (red) and albumin (green) in some cells. Buds were cultured for 5 days without (A) or with 1 μM Dex (B-C) or 1 μM Dex + 10ng/ml OSM (D). Yellow staining indicates co-expression of amylase (red) and albumin (green) in the same cells.

5.5 Retinoic acid affects the development and hepatic metaplasia of pancreatic buds

Retinoic acid has been shown to inhibit the growth of both pancreatic ductal DSL-6A/C1 cells *in vitro* and azaserine-induced foci in the rat pancreas (Brembeck et al., 1998; Roebuck et al., 1984). Recent studies in our lab demonstrate that cell division is necessary for branching morphogenesis and exocrine differentiation. However, endocrine cells can arise from undifferentiated progenitors without cell division (Horb and Slack 2000). Interestingly, when I added all trans-retinoic acid into the culture, we found the concentration of 100nM can inhibit branching morphogenesis and exocrine differentiation (Fig 5.6), but not endocrine differentiation (Fig 5.7). A reduction in the number of CK20 expressing duct cells was also observed.

When we added both Dex and Retinoic acid into culture, I found that the proportion of cells undergoing transdifferentiation to hepatocytes was lower than Dex treated buds, and the inhibition of exocrine differentiation was also reduced (Fig 5.8). Although the mechanism regulating the differentiation of pancreatic exocrine cells needs to be further investigated, it is consistent that hepatocytes were from exocrine cells.



DMSO **RA 100nM** **RA 1uM** **RA 10uM**

Fig 5.6 Effect of different concentrations of all trans-retinoic acid on pancreatic buds. Buds were cultured for 5 days with DMSO or with 100 nM – 10 μM retinoic acids, fixed and stained for amylase (green) and insulin (red).

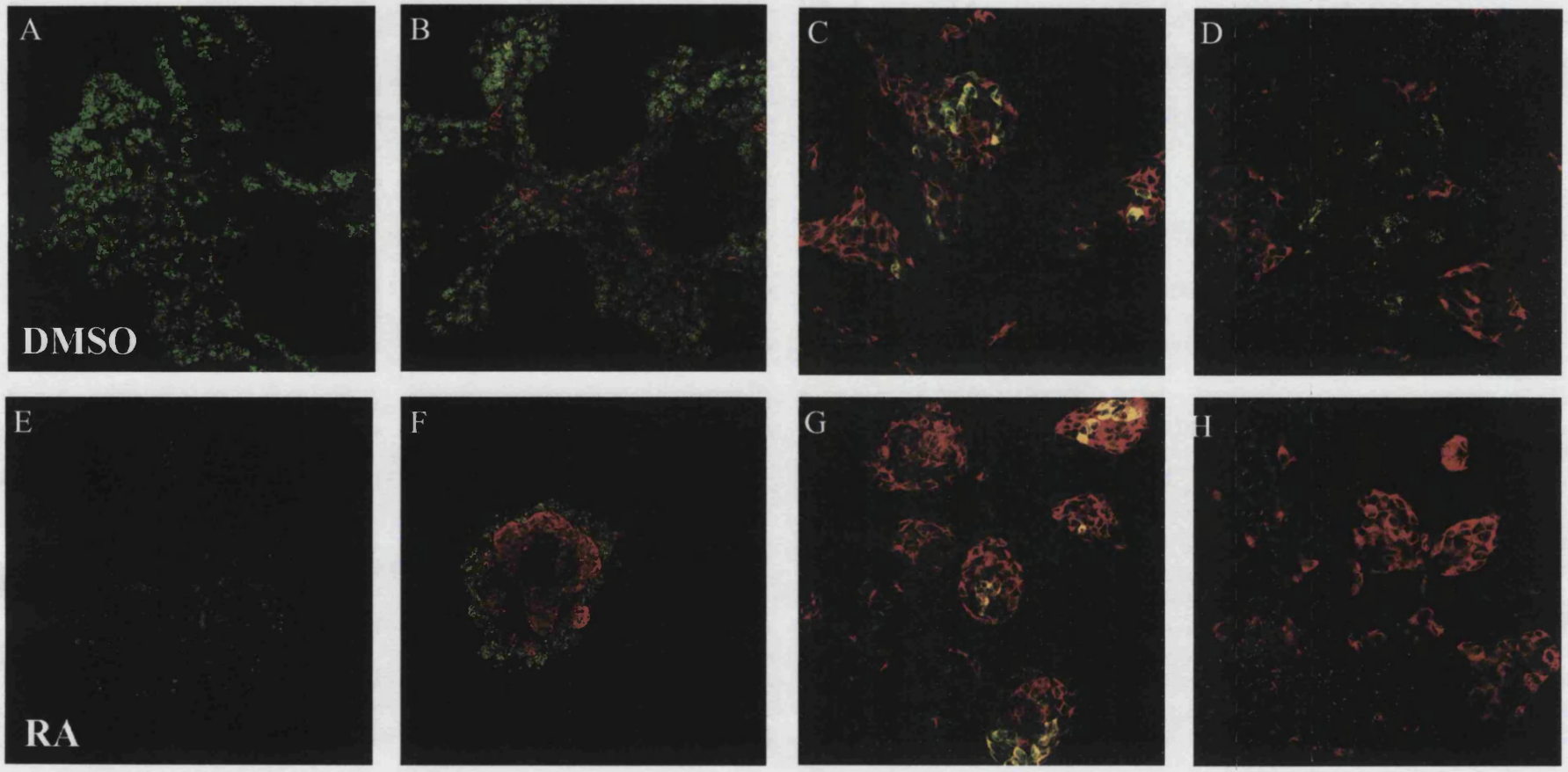

A
DMSO
B
C
D
E
RA
F
G
H
Amylase
Amy / Ins
Gluc / Ins
CK20 / Ins

Fig 5.7 Effect of retinoic acid on pancreatic bud development. Buds were cultured for 3 days (A, E) or 5 days (B-D, F-H) with DMSO (A-D) or with 1 μM retinoic acid (E-H), fixed and stained for (A, E) amylase (B, F) amylase (green)/ insulin (red) (C, G) glucagon (green) / insulin (red) (D, H) CK20 (green) / insulin (red).

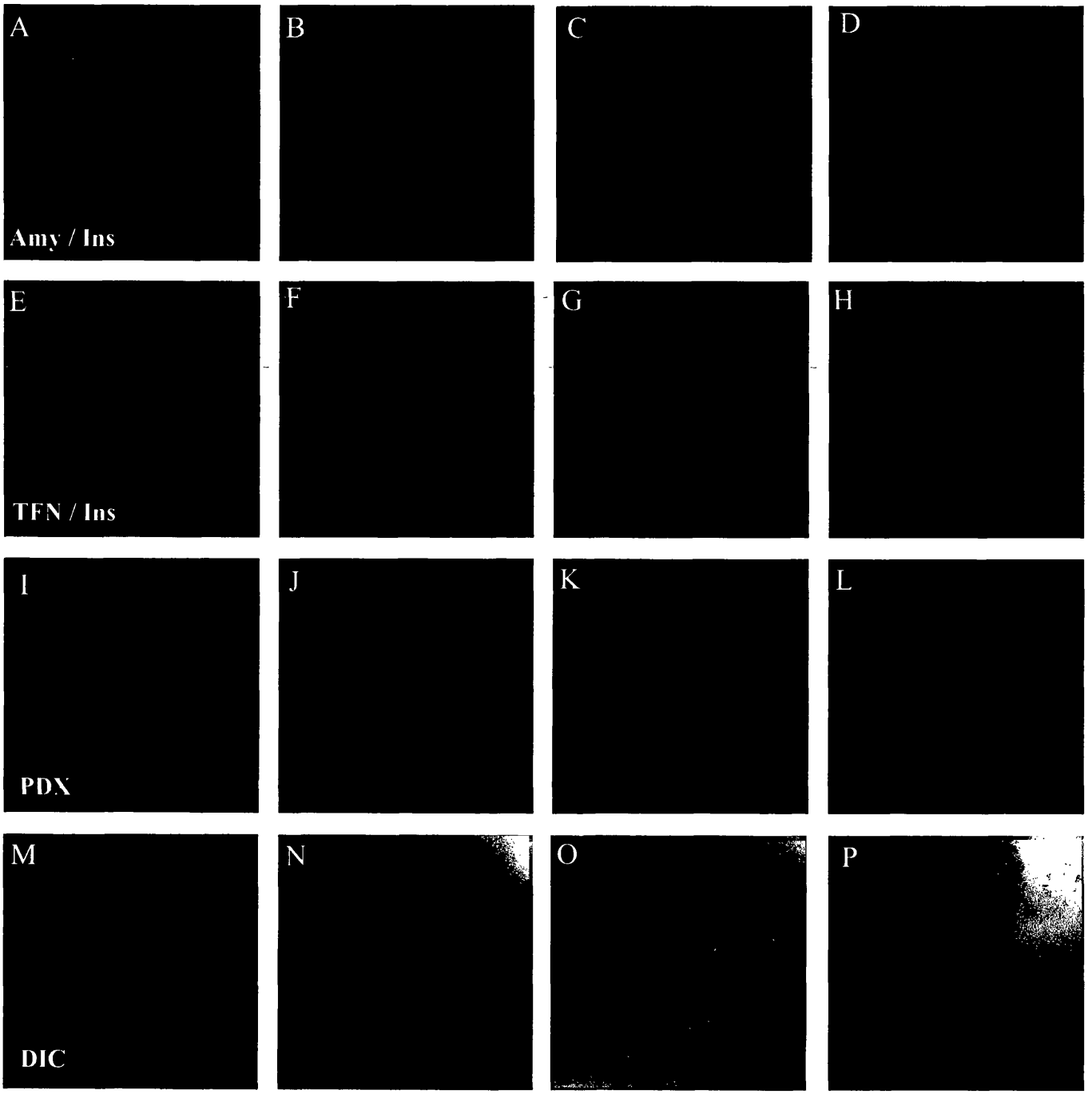

A
Amy / Ins
B
C
D
E
TFN / Ins
F
G
H
I
PDX
J
K
L
M
DIC
N
O
P
DMSO
RA
RA+Dex
DMSO+Dex

Fig 5.8 Effect of retinoic acid on hepatic transdifferentiation in pancreatic buds. Buds were cultured for 5 days with DMSO (A, E, I, M), 1 μM retinoic acid (B, F, J, N), 1 μM retinoic acid + 1 μM Dex (C, G, K, O), or 1 μM Dex + DMSO (D, H, L, P) fixed and stained for (A-D) amylase (green)/ insulin (red) (E-H) transferrin (green) / insulin (red) (I-L) PDX-1.

5.6 Discussion

In this chapter, the results demonstrate that treatment of mouse embryo pancreatic buds with Dex also yields hepatocytes, showing that this effect is not simply a tissue culture artifact but mimics a normal developmental switch. The co-localisation of amylase and albumin in some cells does support the conclusion from our GFP perdurance experiment on the AR42J-B13 cells that at least some of the hepatocytes arise by direct transdifferentiation from pancreatic exocrine cells.

We still cannot exclude the possibility of the stem cells exist in foetal pancreas. Lollis and coworkers have examined foetal pancreases which were obtained from spontaneous abortions (Tsanadis et al., 1995), and found the presence of a characteristic cell-type whose morphology was distinct from islets cells and exocrine pancreas cells was observed in human foetal pancreatic islets. These cells were morphologically similar to hepatocyte-like cells and were comparable to those observed in the experimental models. The result does suggest hepatic precursors exist in the human foetal pancreas. Recent studies performed by Zaret's lab also support the idea. They found there are bipotential precursor cells for pancreas and liver in embryonic development (Deutsch et al., 2001). They suggest that the default fate of the ventral foregut endoderm is to activate the pancreas gene program, and FGF signalling from the cardiac mesoderm diverts this endoderm to express genes for liver instead of those for pancreas. If so, our results may also indicate that the glucocorticoid signalling pathway can regulate or alter the differentiation of pancreatic precursor cells.

CHAPTER 6

GLUCOCORTICOIDS, PANCREATIC DEVELOPMENT AND ADULT DIABETES

6.1 Introduction to diabetes

Diabetes mellitus is a major public health problem, affecting ~5% of the western population, and its treatment consumes more than 10% of the total National Health Service budget in Britain. The chronic complications of diabetes cause enormous human suffering in the form of blindness, kidney failure, limb amputations, and increased risk of coronary artery disease (CAD). There are two common forms of diabetes: Type I diabetes (Insulin dependent diabetes mellitus, IDDM) and Type II diabetes (Non-insulin dependent diabetes mellitus, NIDDM).

Type I diabetes is caused by autoimmune destruction of pancreatic β-cells, leading to a near total deficiency in insulin secretion in response to biological needs. Therefore, the treatment of type I diabetes is complicated all too often by episodes of hypoglycemia or hyperglycemia. Unfortunately, hypoglycemia can cause coma, and hyperglycemia increases the risk of developing diabetic complications such as blindness and kidney failure (Tisch and McDevitt 1996).

Type II is the commonest form of diabetes, accounting for > 90% of diabetic patients. It is caused by two physiological defects: resistance to the action of insulin combined with a deficiency in insulin secretion (Defronzo, 1997). Although the molecular defects causing diabetes are not yet clear, basically it has been found that (1) genetic factors determine the risk of developing type II diabetes, (2) the presence of insulin resistance predicts future development of type II diabetes, and (3) patients become diabetic if they develop insulin deficiency due to failure of pancreatic β-cell (Taylor, 1999). There are a few genes have been identified that cause the common form of type II diabetes or has been found to involve in the mechanism that cause insulin deficiency and insulin resistance.

The insulin gene was the first target to be identified as a type II diabetes gene (Steiner et al., 1990). Mutations in the insulin gene either inhibit conversion of pro-insulin to insulin or reduce the affinity of insulin for its receptor. Recently, several genes have been identified as causes of maturity onset-type diabetes of youth (MODY), an early-onset form of type 2 diabetes transmitted with autosomal dominant inheritance. One example is the glucokinase gene (Bell et al., 1996). Glucokinase phosphorylates glucose to glucose-6-phosphate and plays an essential role in the glucose-sensing mechanism of β cells. Mutations in this gene lead to a partial deficiency of insulin secretion. In other forms of MODY, mutations have been identified in various transcription factors: hepatocyte nuclear factor HNF-1α, HNF-4α, Pancreatic duodenal homeobox-1 (PDX-1/IPF-1/IDX-1), and HNF-1β in MODY1, -3, -4, and -5, respectively (Yamagata et al., 1996a, 1996b; Stoffers et al., 1997; Horikawaet al., 1997). These transcription factors are expressed in pancreatic β cells, and are known to regulate insulin expression. The mutations are believed to cause insulin deficiency by impairing β cell function. However, because HNFs are also expressed in hepatocytes these mutations may cause abnormalities in hepatic glucose metabolism as well.

The insulin receptor, a ligand-stimulated tyrosine kinase, mediates the first steps in insulin action (White and Yenush, 1998). The activated receptor phosphorylates multiple proteins—e.g., insulin receptor substrates (IRS)-1, -2, -3, and -4. Phosphorylation of tyrosine residues in these substrates leads to activation of multiple downstream signalling pathways. For example, activation of phosphatidylinositol (PI) 3-kinase triggers a cascade of serine/threonine protein kinases including protein kinase B and atypical isoforms of protein kinase C, which mediate the metabolic actions of insulin (i.e. stimulation of glucose transport enzymes

in muscle and adipose tissue, and regulation of the expression and activity of key metabolic enzymes). Mutations in the insulin receptor gene (*IR*) cause several uncommon syndromes associated with severe insulin resistance, including leprechaunism and type A insulin resistance (Taylor, 1992), but have also been reported in a small percentage of patients with type 2 diabetes. Amino acid sequence variants have been identified in IRS1, but their role in causing diabetes is controversial (Almind et al., 1996; Imai et al., 1997). Diabetes has not been demonstrated to be associated with mutations in genes encoding other proteins in the insulin action pathway. Indeed, when the p85α subunit of PI 3-kinase was knocked out, the mice developed increased insulin sensitivity and hypoglycemia rather than diabetes (Terauchi et al., 1999).

Type II diabetes is a complex heterogeneous disease with multiple genes contributing to the cause of this polygenic disorder. Defects in the insulin action pathway may be the cause of insulin deficiency in some patients, but there may also be patients in whom there are primary genetic defects that directly impair the machinery for insulin secretion. Type II diabetes is widely believed to be genetically determined, but epidemiological observation has consistently revealed statistical association between poor fetal growth and loss of glucose tolerance in adult life (Phillips 1998; Phillips et al., 1998; Hales 1993; Hill and Duvillie 2000). A possible explanation of these observations is that environmental constraints on fetal growth lead to permanent changes in organogenesis. Although the molecular mechanisms underlying this link are unknown, Seckl and colleagues have provided evidence in animal experiments to show fetal glucocorticoid exposure may play a role (Lindsay et al; 1996b; Nyirenda et al., 1998; Nyirenda and Seckl 1998). This finding is of interest. In the previous chapters, I presented evidence to show that glucocorticoid can induce hepatic

metaplasia in pancreatic bud cultures. In this chapter, firstly I will discuss the correlation between glucocorticoid, fetal programming and diabetes in adult life, and secondly present evidence to show that glucocorticoids can also cause defects in pancreas development, which may lead to the development of type II diabetes in adult.

6.2 Glucocorticoid, fetal programming and adult diabetes

Low birth weight is an important risk factor for type II diabetes in later life. Maturity-onset diabetes of the young has been linked to genetic sequence abnormalities in transcription factors known to be involved in endocrine pancreatic development. These observations suggest that both the maternal environment and the fetal genome can influence the number and/or function of pancreatic beta cells in early life, and that this has life-long implications for postnatal development of diabetes.

Studies in Europe, North America, and the developing world have shown that low birth weight and other indices of abnormal fetal growth in babies are linked with a higher prevalence of glucose intolerance and type II diabetes in adult life (Evidence reviewed in Phillips, 1998). Reduced fetal growth is also associated with a higher prevalence of the metabolic syndrome (in particular, hypertension and vascular disease) and with insulin resistance in adult life. The concept of fetal "programming" has been advanced to explain this phenomenon (Seckl et al., 2000). Fetal adaptations to an adverse intrauterine environment that reduces fetal growth programme results in lifelong physiological changes. These changes in turn predispose to diabetes and the metabolic syndrome. The mechanisms are unknown, but evidence from animal studies and preliminary human evidence suggests that adverse events in early life may influence the neuroendocrine development of the fetus.

One possible link between low birth weight and type II diabetes in adult life is fetal glucocorticoid exposure. Glucocorticoids (corticosterone in rats and mice, cortisol in most other mammals) are produced by the adrenal cortex. In adult mammals, glucocorticoid hormones are involved in control of several physiological processes that maintain homeostasis including coordination of responses to stress (Geley et al., 1996). During development, glucocorticoids have important regulatory functions to prepare the organism for metabolic adaptations necessary for extrauterine life. Normally, the fetus has much lower levels of physiological glucocorticoid than its mother (Campbell and Murphy, 1977). Supra-physiological doses of glucocorticoids retard fetal growth, and human intrauterine growth retardation is associated with elevated cortisol levels (Reinisch et al., 1978).

Fetal glucocorticoid load is, in part, regulated by placental and fetal 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) which catalyses a rapid breakdown of maternal and fetal glucocorticoids into inert 11-keto forms, cortisone and 11-dehydrocorticosterone (Benediktsson et al., 1997). Normally, 11β-HSD2 in the placenta and some fetal tissues is thought to protect the fetus from excess maternal glucocorticoids.

Studies performed by Seckl and colleagues have shown that in rats, birth weight is reduced following either prenatal exposure to the synthetic glucocorticoid dexamethasone, which readily crosses the placenta, or to carbenoxolone, which inhibits 11β-HSD2, the physiological feto-placental "barrier" to endogenous glucocorticoids. (Lindsay et al., 1996) Although the offspring regain the weight deficit by weaning, as adults they exhibit permanent hypertension, hyperglycemia, and increased hypothalamic-pituitary-adrenal axis activity. Moreover, physiological

variations in placental 11β-HSD2 activity near term correlate directly with fetal weight. In humans, 11β-HSD2 gene mutations produce a low birth weight, and some studies show reduced placental 11β-HSD2 activity in association with intrauterine growth retardation. Moreover, low birth weight babies have higher plasma cortisol levels throughout adult life, indicating that hypothalamic- pituitary-adrenal axis programming also occurs in humans (Seckl 2000).

Administration of dexamethasone (a poor substrate for 11β-HSD2) to pregnant rats in the last week of pregnancy reduces birth weight by 10%, and produces adult fasting hyperglycemia, reactive hyperglycemia, and hyperinsulinemia on oral glucose loading. In the adult offspring of rats exposed to dexamethasone in late pregnancy, hepatic expression of phosphoenolpyruvate carboxykinase (PEPCK) mRNA (and activity) is increased by 60% (Nyirenda et al., 1998). These observations indicate that programmed PEPCK overexpression in liver promotes gluconeogenesis which may cause glucose intolerance in adulthood. This animal model of glucocorticoid programming may provide a chance to explain the association between fetal growth and subsequent disorders in adult life including type II diabetes.

As I mentioned earlier in this chapter, type II diabetes may be caused by either or both of two physiological defects: resistance to the action of insulin and defects in insulin secretion. The aim of the following sections of this chapter was to determine whether glucocorticoid exposure during embryogenesis might cause defects in pancreatic development and whether hepatic metaplasia of the pancreas also occurs.

6.3 Glucocorticoid and abnormal development of the pancreas

In the previous chapters, I presented evidence to show that pancreatic cells can be converted into hepatocytes by glucocorticoid treatment. This occurs both in a pancreatic cell line, AR42J-B13, and in organ cultures of pancreatic buds from mouse embryos. In order to investigate whether treatment with glucocorticoid in mid-late gestation can affect the pancreas development itself, further experiments were carried out. Different concentrations of Dex (from 1 nM to 1 μM) were added to the 2d pancreatic bud cultures and incubated for 5 days. As expected, there were some hepatocyes formed in the 1 μM Dex treated culture as judged by transferrin staining (Fig 6.1 A-E). Interestingly, we can even find transferrin positive cells in buds treated with 1 nM Dex (Fig 6.1A). This is slightly different from the results I obtained in AR42J-B13 cells (Chapter 3).

The cultures were analysed for the expression of two genes-insulin and Pancreatic duodenal homeobox-1 (PDX-1, also known as insulin promoter factor-1 (IPF-1)). In chapter 5, I have shown that there is a large number of insulin expressing cells forming in control 7-day bud culture, and some of the cells start to form an islet-like structure. Treatment with Dex caused the significant reduction of both insulin and PDX-1 expression (Fig 6.1F-O). If the effect of Dex persists, it might cause insulin deficiency in late life, which might lead to type II diabetes.

In order to analyse whether the effect can persist after removal of glucocorticoid from medium, I set up a time course experiment, and added 1 μM Dex to the buds at day 2, and cultured for five days, then removed Dex and cultured for a further 4 days. Representative fields are shown in Fig 6.2. The early expression of PDX1 is not much affected by the Dex, but the late expression is greatly reduced and remains low 4 days after Dex withdrawal. PDX-1 normally has two phase of

expression: the early phase in the whole pancreatic bud region and the later phase in the β cells where it serves as a positive regulator of insulin transcription. The results shown here suggest that glucocorticoids reduce the insulin expression and that the effect may outlast the period of treatment.

After removal of Dex from culture for 4 days, we still can find some cells expressing transferrin (Fig 6.2 O). This result suggests the hepatic metaplasia is an irreversible process which is supported by observation in AR42J-B13 cells (see Chapter 3.8) There were some cells re-expressed insulin and PDX-1 (Fig 6.2 F, L), but either the organisation of pancreas or the ratio of cells has been altered by Dex treatment.

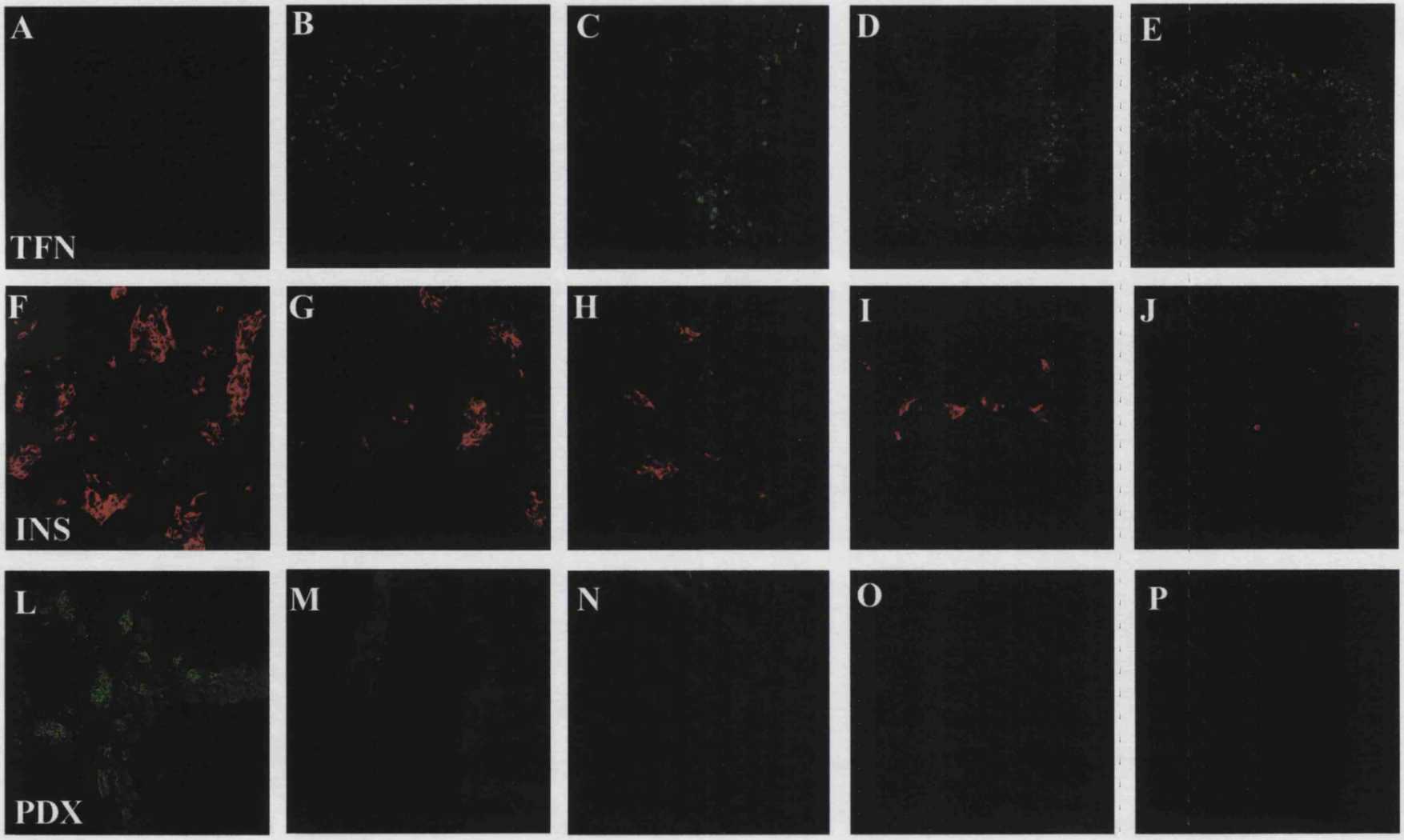

A
TFN
B
C
D
E
F
INS
G
H
I
J
L
PDX
M
N
O
P
Control
Dex 1nM
Dex 10nM
Dex 100nM
Dex 1uM

Fig 6.1 Effect of different concentrations of Dex (0, 1,10,100,1000 nM) on pancreatic buds. Buds were cultured for 5 days without or with Dex, fixed and stained for transferrin (A-E), insulin (F-J) or PDX-1 (L-P)

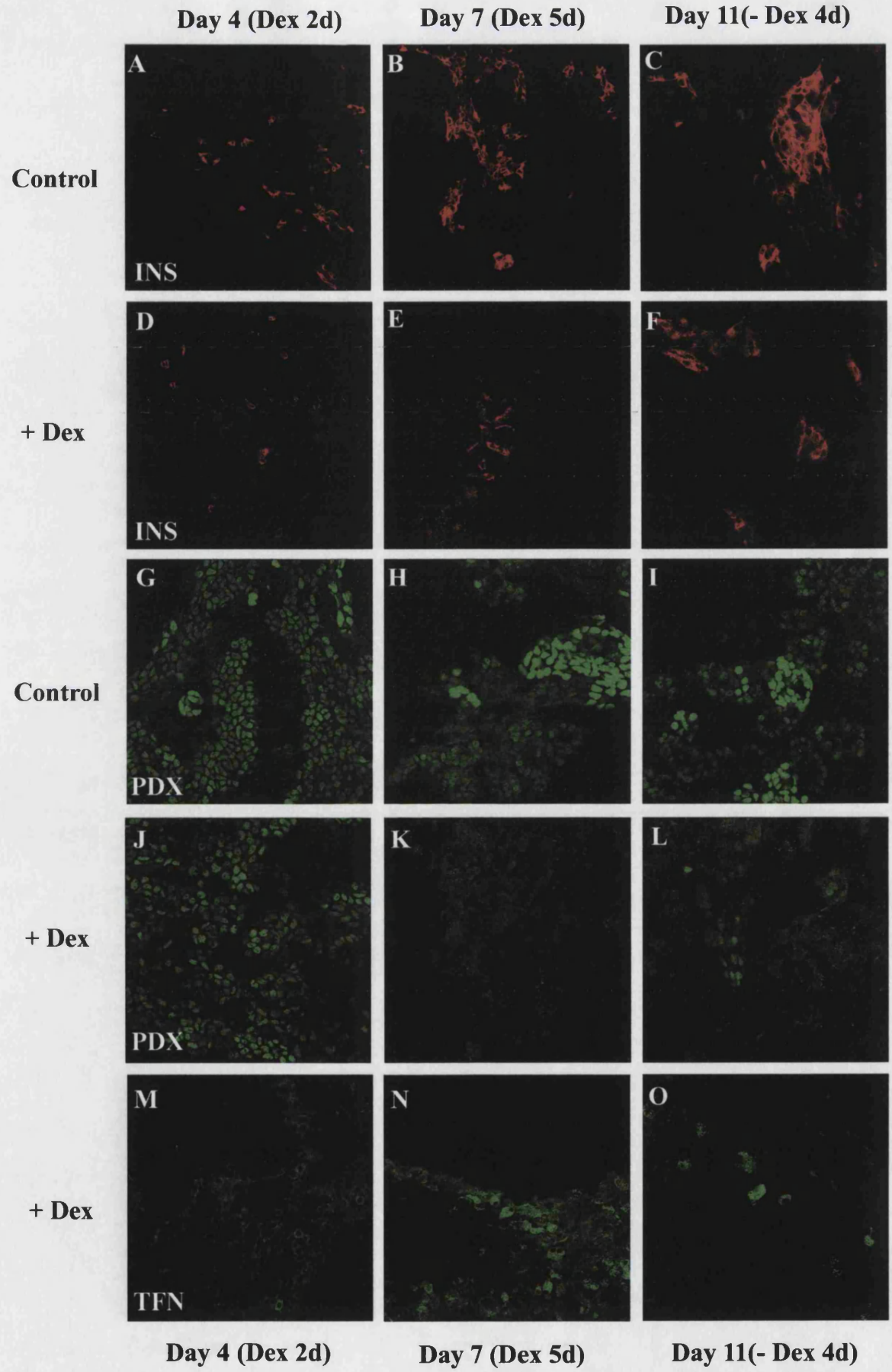

Day 4 (Dex 2d)
Day 7 (Dex 5d)
Day 11(- Dex 4d)
Control
+ Dex
Control
+ Dex
+ Dex
A
B
C
INS
D
E
F
INS
G
H
I
PDX
J
K
L
PDX
M
N
O
TFN
Day 4 (Dex 2d)
Day 7 (Dex 5d)
Day 11(- Dex 4d)

Fig 6.2 Time-course of insulin and PDX-1 expression with or without 1 μM Dex. Buds were isolated from E11.5d mice embryo, and cultured for 2 days without treatment. Some buds were treated with 1 μM Dex for 2 days (D, J, M), or 5days (E, K, N). For F, J and O, buds were treated with 1 μM Dex for 5 days, and then Dex was removed and cultures was maintained in control medium for a further 4 days. Cultures were fixed at day 4, day 7 and day 11, and stained for insulin (A-F), PDX-1 (G-L) or transferrin (M-O)

6.4 Suppression of insulin via induction of C/EBPβ

Glucocorticoids are diabetogenic hormones because they decrease glucose uptake and increase hepatic glucose production (Delaunay et al., 1997). In addition, they may directly inhibit insulin release. Glucocorticoid has been shown to inhibit insulin expression in cultured pancreatic islet cells (Fernandez-Mejia et al., 1999; Seufert et al., 1998). Importantly, previous studies performed by Habener and colleagues identified the basic leucine zipper transcription factor C/EBPβ as an inhibitor of insulin gene transcription in pancreatic beta cells and is also upregulated in animal models of diabetes mellitus. Concomitant with a downregulation of PDX-1 and insulin expression, C/EBPβ is upregulated in association with the manifestation of hyperglycemia during the development of diabetes in the Zucker diabetic fatty (fa/fa) rat (Seufert et al., 1998). These findings suggest a role for glucocorticoids and C/EBPβ the development of diabetes.

In previous chapters, we have shown that dexamethasone can induce C/EBPβ which plays a key role in the formation of hepatic metaplasia *in vitro* and *ex vivo*. In this chapter, we also find the formation of hepatic metaplasia accompanied by the reduction of insulin expression in pancreatic buds. In order to determine whether induction of C/EBPβ by dexamethasone is involved in the down-regulation of insulin, I decided to examine the response of RIN-5F cells. RIN-5F is a subclone drived from RIN-m cells, widely used pancreatic β-cell lines that can express and produce insulin. Here the results show that treatment of 1uM of Dexamethasone for 4 days is sufficient to suppress insulin and increase C/EBPβ expression in RIN-5F cells (Fig 6.3A-C). After 6 days of Dex treatment, the expression of insulin was almost gone (Fig 6.4 C).

When I pre-treated the cells with glucocorticoid inhibitor-RU486, the expression of C/EBPβ was suppressed and the insulin expression maintained (Fig 6.3 D).

In order determine whether C/EBPβ is a key gene in the reduction of insulin expression, I transfected LIP (the inactive form of C/EBPβ) and LAP (the active form of C/EBPβ) into RIN-5F cells. As expected, the transfection of LIP didn't cause the reduction of insulin and the transfection of LAP was sufficient to inhibit the expression of insulin (Fig 6.5 B, C). These findings suggest the induction of C/EBPβ by dexamethasone play the major role in suppression of insulin. Interestingly, the expression of PDX-1 also is partially inhibited by dexamethasone (Fig 6.3 E-G).

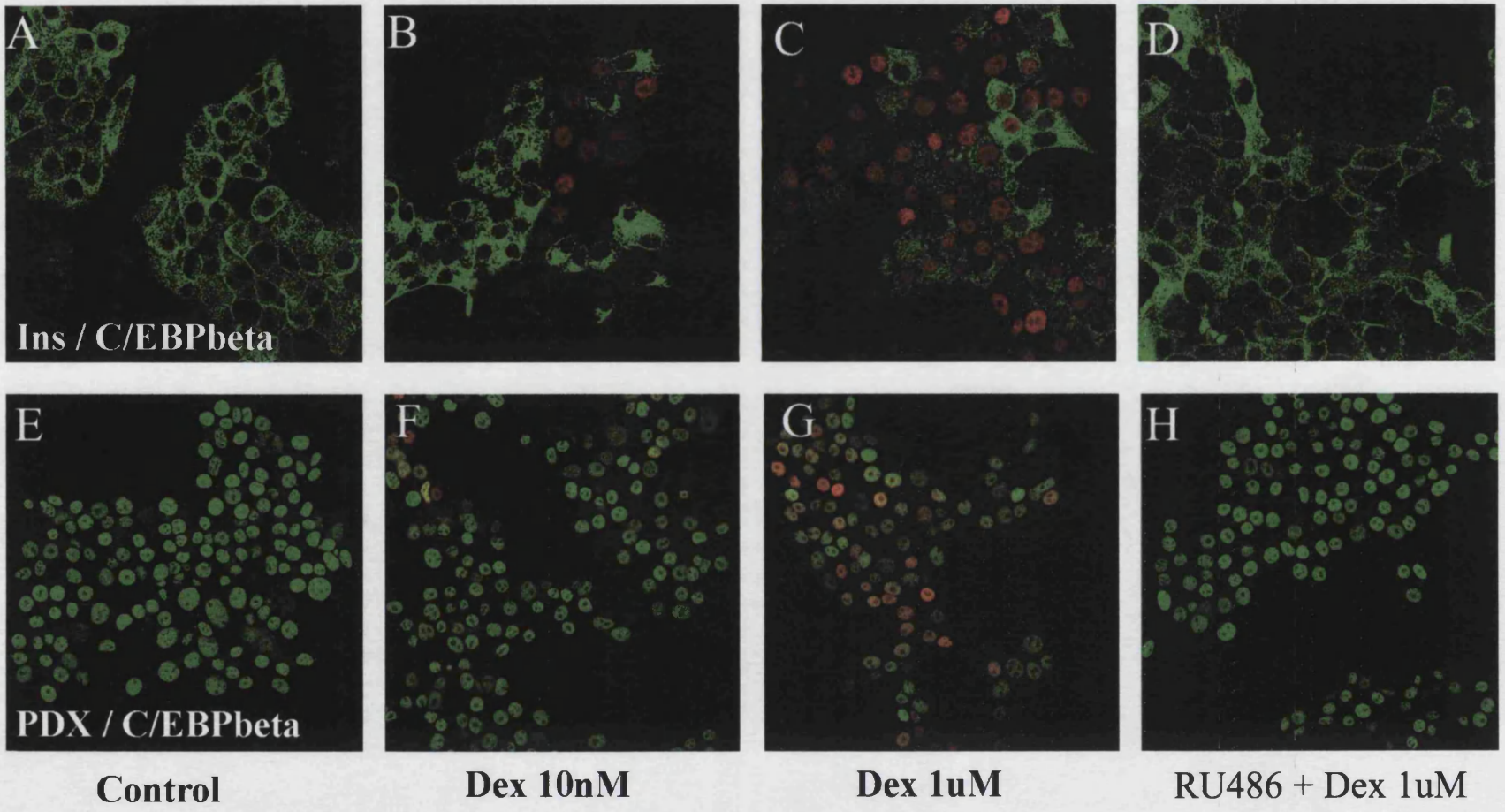
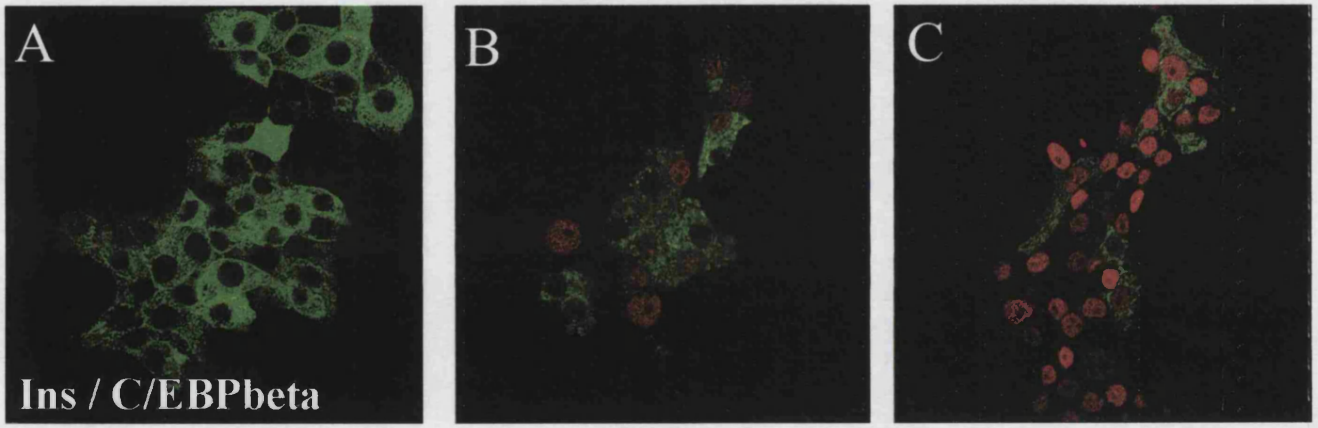

A
B
C
D
Ins / C/EBPbeta
E
F
G
H
PDX / C/EBPbeta
Control
Dex 10nM
Dex 1uM
RU486 + Dex 1uM

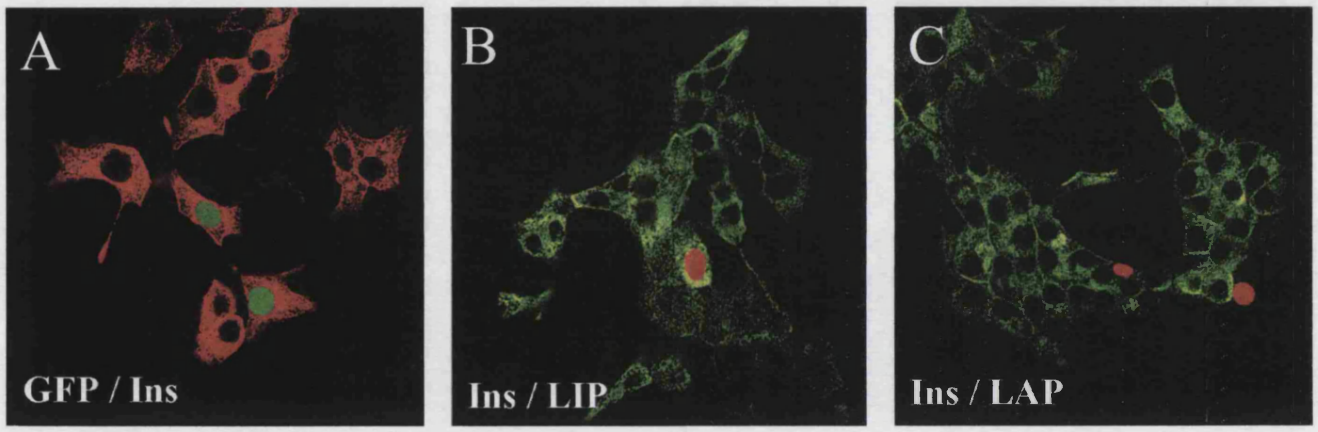
Fig 6.3 Dexamethasone suppresses insulin and increases C/EBPβ expression in RIN-5F cells. Cells were incubated with 10 nM or 1 μM Dex for 4 days with or without the pretreatment of 2.5 μM RU486. Cells were dual-stained with (A)-(D) anti-insulin (Green) and anti-C/EBPβ (Red) antibodies or (E)-(H) anti-PDX (Green) and anti-C/EBPβ (Red) antibodies.



Control **Dex 4d** **Dex 6d**

Fig 6.4 Time-course of insulin expression in RIN-5F cells treated with Dex.

RIN-5F cells were treated with 1 μM Dex for 4days (B) and 6 days (C), and then fixed and stained for anti-insulin (Green) and anti-C/EBPβ (Red) antibodies.



CMV-nucGFP **CMV-LIP** **CMV-LAP**

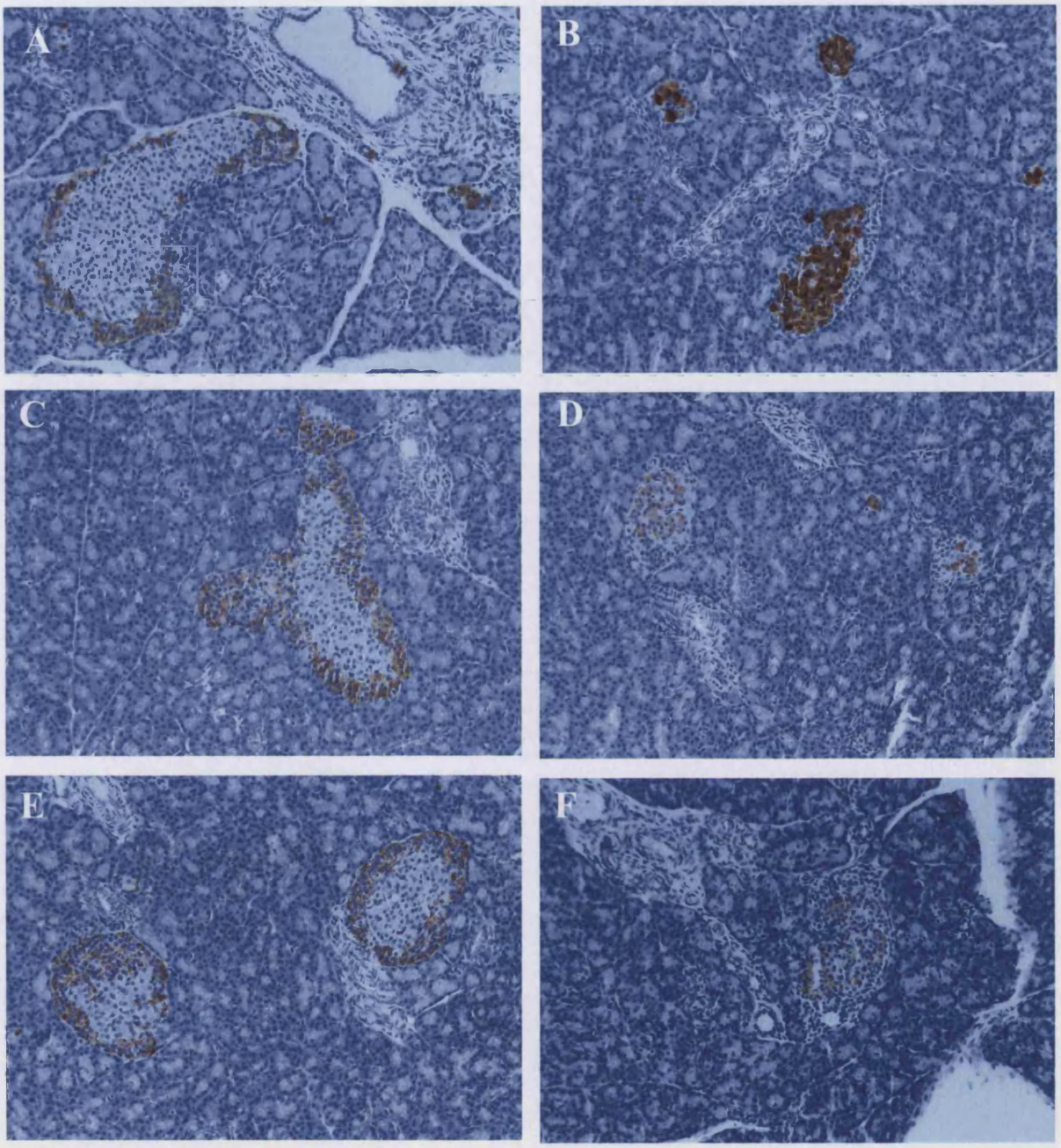
Fig 6.5 Inhibition of expression of insulin by LAP

RIN-5F cells were transfected with CMV-nucGFP, CMV-LIP (inactive form of C/EBPβ) and CMV-LAP (active form of C/EBPβ). After 72 hrs of transfection, cells were fixed and dual-stained for (A) anti-insulin (Red) / anti-GFP (Green) antibodies (B)-(C) anti-insulin (Green) / anti-C/EBPβ (Red). (i.e. LIP or LAP can be detected by immunohistochemistry because the C/EBPβ antibody is raised to the C-terminus. LIP or LAP transfected cells are intensely stained)

6.5 Formation of abnormal islets *in vivo*

To study whether exposure of glucocorticoid in the late gestation can cause the alteration of pancreatic organisation and hepatic metaplsia *in vivo*, we obtained the glucocorticoid treated rats' pancreas from Prof. J. R. Seckl. The procedure was performed as described previously (Nyirenda et al, 1998). Briefly, mothers were treated throughout the 3rd week of gestation (from day 15 to day 21-22 (depending on when pups were born)), and then the offspring were sacrificed 3 weeks postnatally. Adult offspring have been shown to display hyperglycemia.

Here, we investigate the distribution of α- and β-cells in the islets via the presence of insulin and glucagon by immunohistochemistry. Glucagon-secreting α-cells are normally distributed around the periphery of the islet while the insulin-secreting β-cells represent the majority of the islet cells and fill its core (Fig 6.6A, B). In glucocorticoid treated animals, the distribution of α-cells was altered. Occasionally, glucagon-secreting α-cells can be found to penetrate deep into the islet (Fig 6.6C, E). In addition, there were fewer numbers of insulin positive cells in the islet, and staining for insulin was less intense (Fig 6.6 D, F) compared to control sample (Fig 6.6B). The results suggest a permanent reduction of β- cells mass occurred in these Dex treated rats which may cause imbalance of glucagon and insulin secretion. .



Glucagon **Insulin**

Fig 6.6 Glucagon or insulin in sections of pancreas from control rats (A, B) or rats treated with Dex during the late gestation (C-F). A, C, E glucagon staining; B, D, F insulin staining

6.6 Formation of hepatocyte-like cells

Since the alteration of pancreatic organisation can be observed in the offspring of the glucocorticoid-treated dams which is similar to the result we obtained in copper-deficient rats (Tosh et al., 2002, Data not shown), I decided to examine whether pancreatic hepatocytes exist in the animals. We performed histological examination of H&E stained sections of pancreas from 5 animals. There were some eosinophilic staining cells in one animal which is a feature typical of hepatocytes (Fig 6.6C-E). In order to determine whether these cells are hepatocytes, the neighbouring sections were stained with transferrin and albumin antibodies. The result showed that these eosinophilic staining cells did indeed express transferrin and albumin (Fig 6.7). Normally, albumin also exists in the circulation system, so some blood vessels have been stained positive for in the control (Fig 6.8).

The finding of hepatocyte-like cells existed in these glucocorticoid treated animal supports our results *in vitro* and ex *vivo*. That is to say, glucocorticoids could possibly induce hepatic metaplasia *in vivo*. Unfortunately, like most in vivo experiments, it is very difficult to determine the origin of these hepatocyte-like cells, and we are so far unable to conclude the association of the hepatic metaplasia and developing glucose intolerance in this animal model.

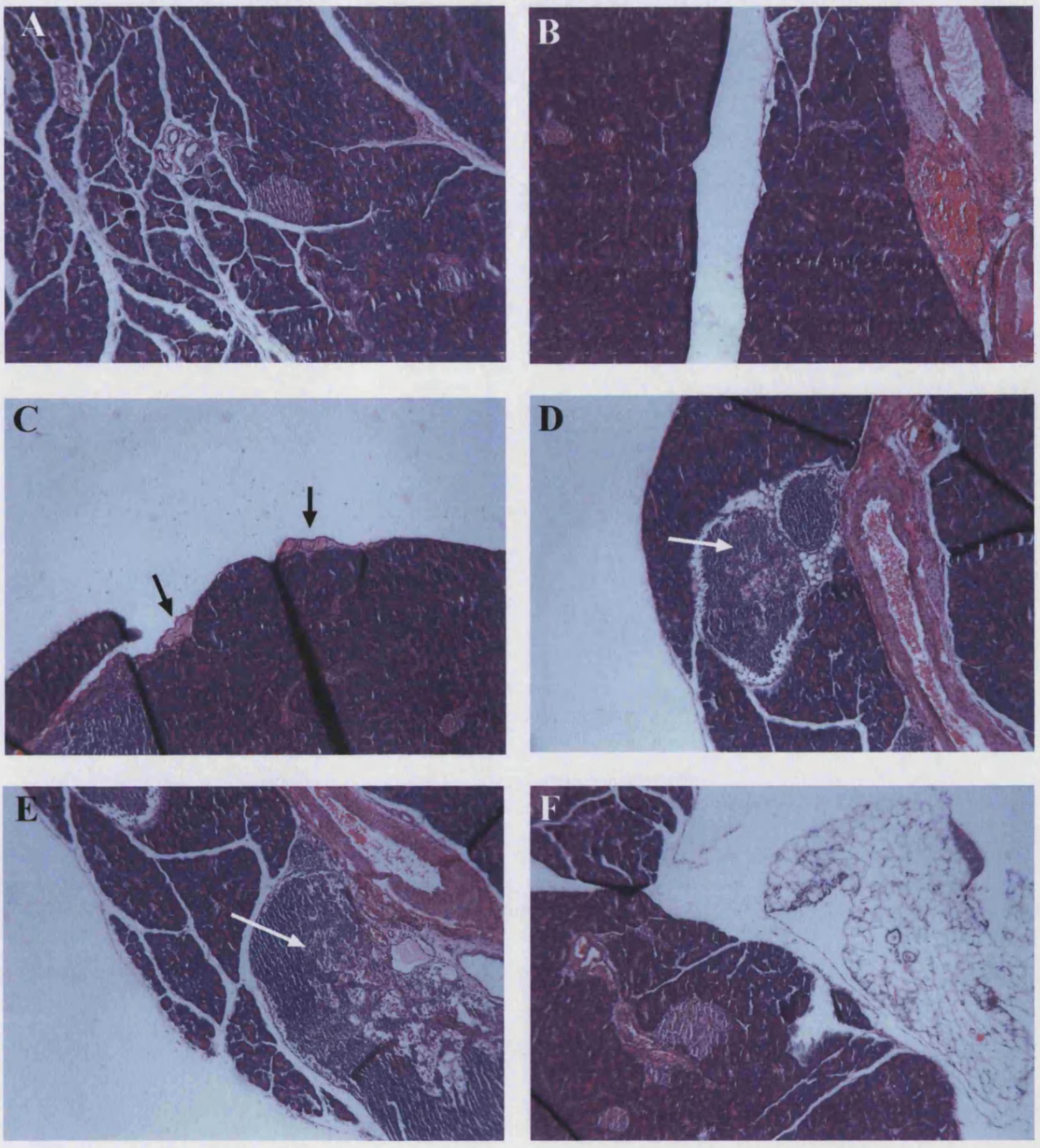

A
B
C
D
E
F

Fig 6.7 Haemotoxylin and eosin staining of pancreases from either control rats (A-B) or treated with Dex during the late gestation (C-F). (C) show eosinophilic staining cells in the edge. (D, E) shows eosinophilic staining cells in lymphoid-like tissue within pancreas. (F) Some fat tissue can be found in the pancreas.

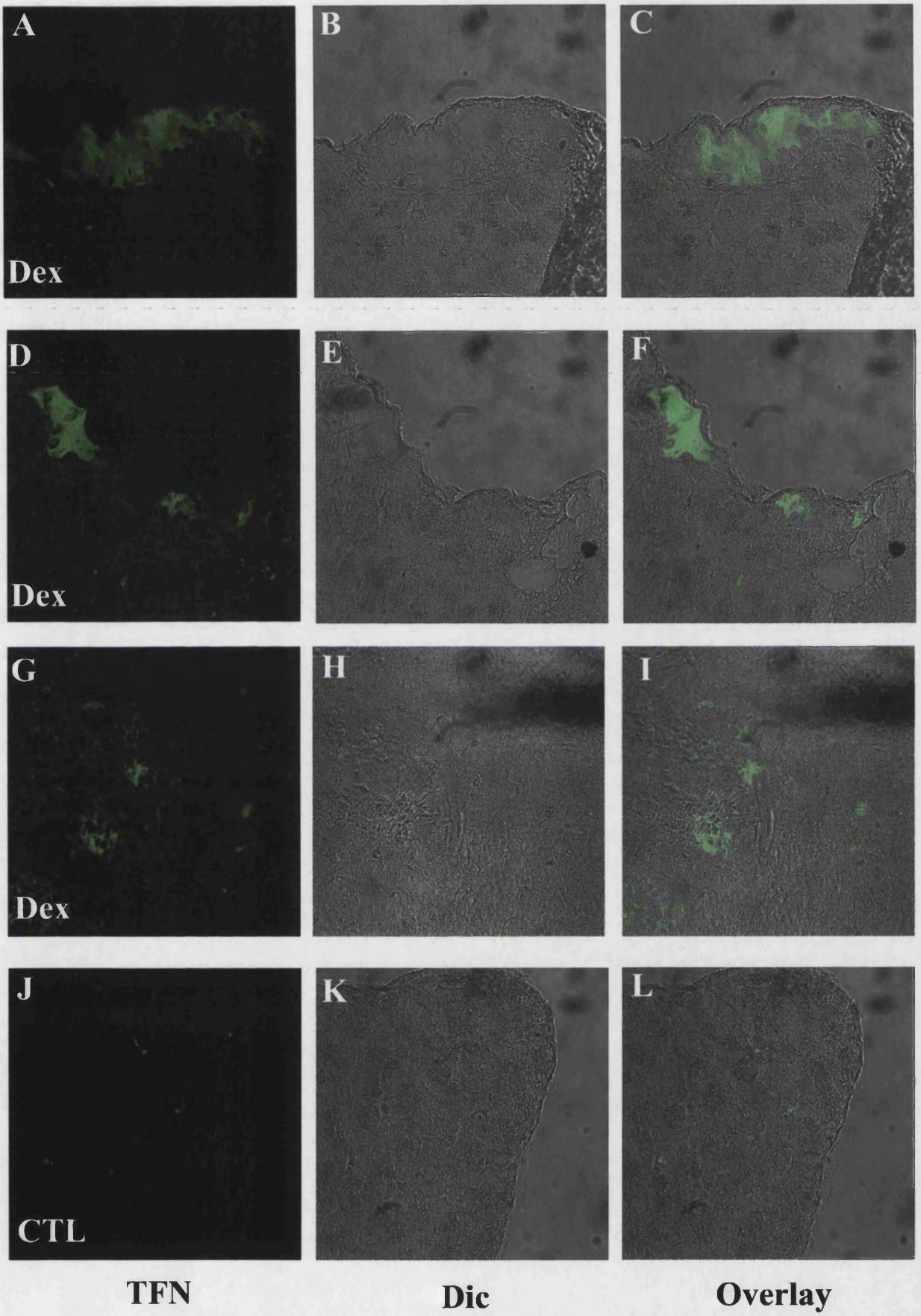

A
B
C
Dex
D
E
F
Dex
G
H
I
Dex
J
K
L
CTL
TFN
Dic
Overlay

Fig 6.8 Eosinophilic staining cells expressed transferrin. Sections from either rats treated with Dex during late gestation (A-I) or control rats (J-L) stained with anti-transferrin antibodies. (A, D) show eosinophilic staining cells in the edge expressed transferrin. (G) shows eosinophilic staining cells in lymphoid-like tissue also expressed transferrin.

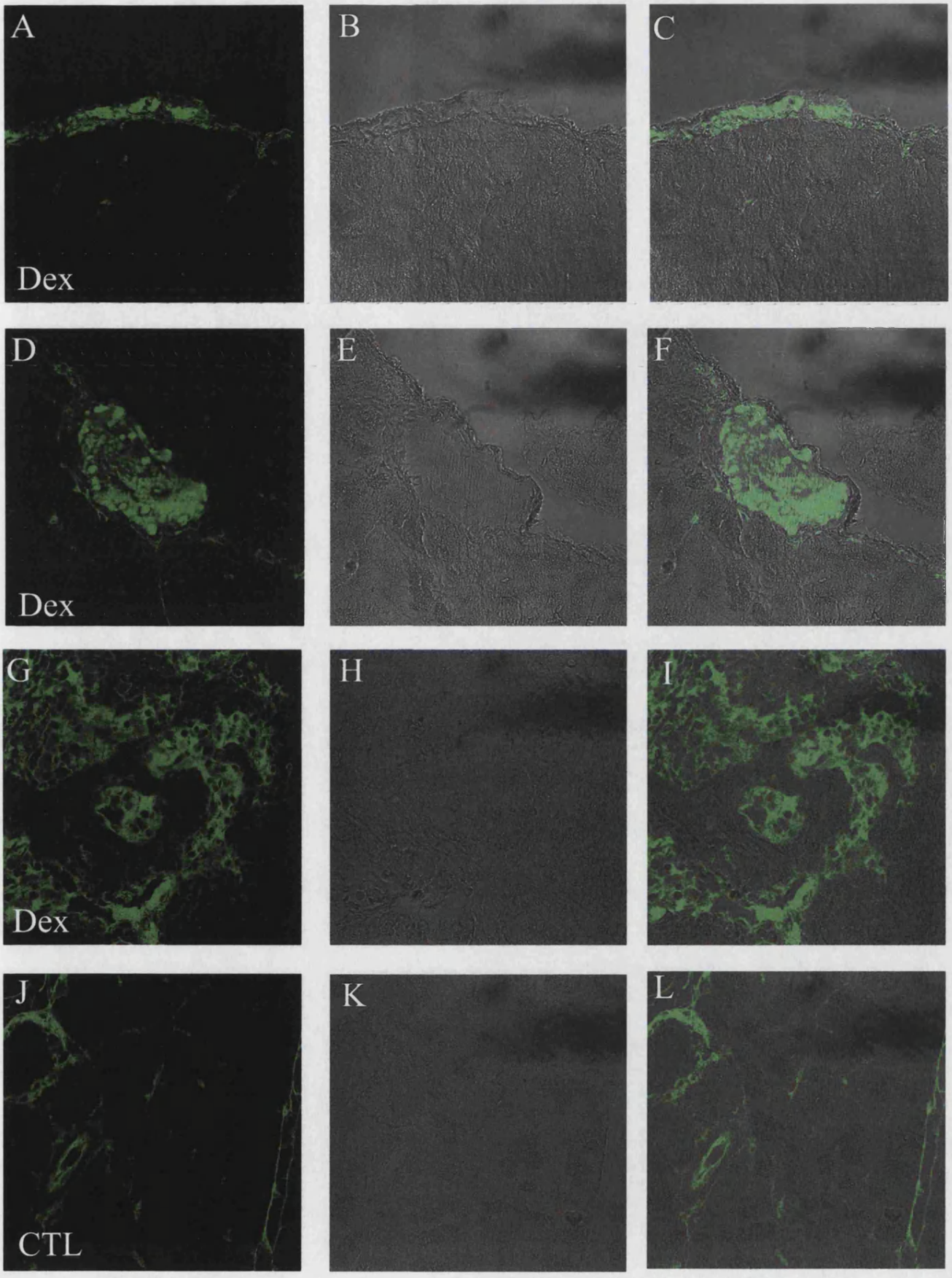

A
Dex
B
C
D
Dex
E
F
G
Dex
H
I
J
CTL
K
L
Alb
Dic
Overlay

Fig 6.9 Eosinophilic staining cells expressed albumin. Sections from either rates treated with Dex during the late gestation (A-I) or control rats (J-L) were stained with anti-albumin antibodies. (A, D) show eosinophilic staining cells in the edge expressed albumin. (G) shows eosinophilic staining cells in lymphoid-like tissue also expressed albumin. (J) Blood vessels have been stained positive for albumin, which serve as a positive control.

6.7 Discussion

We demonstrate in this chapter that the treatment with 1 nM Dex is sufficient to reduce insulin and PDX-1 expression in mouse embryo pancreatic buds. The results indicate that the fetal pancreas may be very sensitive to glucocorticoids, and it could be the reason why the fetus has much lower levels of physiological glucocorticoid than the adult. Recent evidence provided by Bre'ant and colleagues also supports our finding (Blondeau et al., 2001). They show that undernutrition significantly increased maternal and fetal corticosterone level. Twenty-one-day-old fetuses with undernutrition showed growth retardation and decreased pancreatic insulin content. What is more, adrenalectomy and subcutaneous corticosterone implants in their dams prevented the maternal corticosterone increase and restored fetal β-cell mass. Their data also suggest a negative role of glucocorticoids in fetal β-cell development.

Hattersley and Tooke made a "fetal insulin hypothesis" to explaine the association of low birthweight with diabetes and vascular disease (Hattersley and Tooke, 1999). They propose that genetically determined insulin resistance results in impaired insulin- mediated growth in the fetus as well as insulin resistance in adult life. There is evidence to support this hypothesis (Hill, 1976). Human disorders where excessive insulin secretion and beta cell hyperplasia are present result in fetal overgrowth. Experimental models confirm an important anabolic effect of insulin *in utero*. In contrast with this data is the evidence of intrauterine growth retardation occurring in association with insulinopenia or total absence of fetal insulin. Without the anabolic effect of insulin, no amount of substrate will induce optimal growth. It is evident from both the clinical and experimental data that sensitivity to insulin (probably individual receptor sites) develops in the latter stages of pregnancy.

Insulin secretion by the fetal pancreas in response to maternal glucose concentrations is a key growth factor. Monogenic diseases that impair sensing of glucose, lower insulin secretion, or increased insulin resistance are associated with impaired fetal growth. In this chapter, we found the exposure of fetal pancreas to glucocorticoid could significantly affect the formation of β cells, or the expression of insulin. And the reduction of fetal insulin plays a role to cause the fetal retardation.

PDX-1 is a homeodomain protein. In mouse embryos, PDX-1 expression is restricted to the developing pancreatic anlagen and is initiated when the foregut endoderm is committed to a pancreatic fate. Edlund and her colleagues showed that mice homozygous for a targeted mutation in the *pdx-1* gene selectively lack a pancreas (Jonsson et al., 1994). The mutant pups survive fetal development but die within a few days after birth. The gastrointestinal part and all other internal organs were normal in appearance. No pancreatic tissue and no ectopic expression of insulin was detected in mutant embryos and neonates. These findings show that PDX-1 is needed for the formation of the pancreas and suggest that it acts to determine the fate of common pancreatic precursor cells and/or to regulate their propagation. In the adult mouse pancreas, PDX-1 is selectively expressed in the β-cells and binds to and transactivates the insulin promoter (Habener and Hussain 2001). β-cell specific inactivation of the mouse *Ipf1/pdx1* gene results in loss of the beta-cell phenotype and maturity onset diabetes (Ahlgren, 1998). Here, we also showed PDX-1 was down-regulated by glucocorticoid. Montminy and co-workers have performed experiments in HIT cells which showed that glucocorticoid can repress PDX-1 gene expression by interfering with HNF-3β activity on the islet-specific enhancer (Sharma et al., 1997). As a result, we believe reduction of PDX-1 in the late gestation by glucocorticoids could play a

role to form the abnormal pancreas and develop diabetes in postnatal life.

In previous chapters, we have shown that dexamethasone can induce C/EBPβ which plays a key role in the formation of hepatic metaplasia *in vitro* and *ex vivo*. Here, we also show that Dex can induce the expression of C/EBPβ in RIN-5F cells. The transfection of LAP, the constitutively active form of C/EBPβ is sufficient to suppress insulin expression. Previous studies performed by Habener and colleagues also show that after exposure to high glucose concertration, the β-cell line HIT-T15 and INS-1 express high level of C/EBPβ which directly binds to rat insulin I promoter and inhibits the transcription (Lu et al., 1998). The result also supports the idea that expression of C/EBPβ can inhibit insulin expression.

Although there is no evidence to suggest that hepatic metaplasia plays a role in the development of type II diabetes, the liver plays a central role in the control of glucose homeostasis and is subject to complex regulation by substrates, insulin, and other hormones. Insulin resistance in the liver has been suggested to be a later factor in the development of hyperglycemia, with increased hepatic glucose production tightly correlated with fasting hyperglycemia in type 2 diabetic individuals (DeFronzo, 1997). Recent evidence provided by Kahn and colleagues has shown loss of direct insulin action in mice caused dramatic insulin resistance, severe glucose intolerance, and a failure of insulin to suppress hepatic glucose production and to regulate hepatic gene expression (Michael et al., 2000). Thus, the result suggests that insulin signalling in the liver is critical in regulating glucose homeostasis and maintaining normal hepatic function. In present studies, we show that glucocorticoid treatment reduces the expression of insulin during embryogenesis and/or in postnatal life. The defect in insulin signalling would probably disrupt the glucose homeostasis and cause abnormal

hepatic function. What is more, the formation of pancreatic hepatocytes may also be involved. Although, in the present studies, we cannot provide the further evidence to support this hypothesis, it at least provides an additional view to uncover the mechanism which may cause type II diabetes in later life.

CHAPTER 7

FINAL DISCUSSION AND FUTURE PROSPECTS

Numerous examples of transdifferentiation exist but perhaps one of the most interesting is the appearance of hepatocytes in the pancreas. Foci of hepatocytes have been shown to be induced in the pancreas of rats, mice or hamsters following various experimental procedures. I demonstrate in the present study the ability of the synthetic glucocorticoid dexamethasone to induce the formation of hepatocytes from pancreatic cells. The fact that treatment of mouse embryo pancreatic buds also yields hepatocytes shows that this effect is not simply a tissue culture artifact but mimics a normal developmental switch.

In the AR42J-B13 cells, the addition of Dex induces morphological change. The cells begin to flatten onto the substratum within 48-72hrs. The morphological change is associated with the gain of expression of liver markers. B13 cells induced to undergo transdifferentiation to hepatocytes with Dex begin to express a_1-antitrypsin, glucose-6-phosphatase (G6Pase), phenol sulphotransferase and transferrin. Later (around 7 days) the cells begin to express albumin. Embryonic pancreatic buds cultured with Dex also begin to express liver markers suggesting that the conversion of pancreatic cells to hepatocytes by glucocorticoid is physiologically relevant.

A variety of cell types have been proposed to give rise to hepatocytes including exocrine and endocrine cells as well as the pancreatic ductular system. Although several experimental models support the concept that cells capable of transdifferentiating to hepatocytes exist in the pancreas, there is no consensus as to which are the real progenitor cells. In order to distinguish where hepatocytes were derived from exocrine cells a lineage experiment was designed based on the perdurance of green fluorescent protein. The exocrine-specific elastase promoter was used to drive green fluorescent protein and transfected into B13 cells. This experiment

clearly shows that GFP and liver markers can be detected in the same cell. It indicates some of hepatocytes must have arisen from cells which had an active elastase promoter (differentiated exocrine cells). Such a direct conversion between one differentiated cell type (exocrine cell) and another (hepatocyte) is a true transdifferentiation event.

The conversion of pancreatic cells to hepatocytes provides the novel opportunity to study the molecular basis of transdifferentiation and perhaps identify the master switch gene for pancreas to liver in the process. To understand the molecular basis of the switch several approaches have been taken. AR42J-B13 cells were examined for the expression of various liver-enriched transcription factors and it is apparent that those examined are already expressed in the pancreatic cell line. In the normal pancreas, many of the LETFs are also already expressed albeit in different cell types. However, C/EBPβ was not normally expressed in B13 cells but is induced shortly after Dex-treatment. When C/EBPβ was transfected into B13 cells, the expression of amylase was reduced and some liver markers were expressed suggesting that this transcription factor is important in the conversion of pancreatic cells to hepatocytes. To test this further, when the dominant negative form of C/EBPβ (termed liver inhibitory protein or LIP) was transfected into B13 pancreatic cells transdifferentiation was inhibited. This evidence suggests that C/EBPβ may indeed be the master switch gene for the liver to pancreas transdifferentiation.

Low birth weight is an important risk factor for type II diabetes in later life. In the present studies, I also show that glucocorticoids can both abnormal cause pancreatic development and hepatic metaplasia. Significant reduction of insulin expression was observed in Dex treated pancreatic buds, RIN-5F cells and in the pancreas of rat offspring. Pancreatic hepatocytes were also found in Dex treated pancreatic buds and one example of a pancreas exposed to Dex *in utero*. The evidence

suggests glucocorticoid not only can induce hepatic metaplasia, but it might also play a negative role in regulating fetal growth and pancreatic development.

As AR42J-B13 cells resemble a pluripotent pancreatic cell, so precursor cells might exist. In this study, I cannot provide sufficient evidence to exclude the possibility. Although we do show that at least some of the hepatocytes are formed directly from exocrine-like cells with no intervening cell division, we cannot ignore the possibility that hepatocytes arises from stem cells. Recent evidence show that tissue-specific stem cells can give rise to cells of heterologous lineages (Goodell 2001). For example, hepatocytes can differentiate from hematopoietic stem cell populations. Recent evidences also show that tissue-specific stem cells can give rise to cells of heterologous lineages (Goodell 2001). For example, hepatocytes can differentiate from hematopoietic stem cell populations. Recent lineage-marking studies of mice after bone marrow transplantation have challenged the view that hepatocytes must be derived from the endoderm or endoderm-derived cells (Petersen et al., 1999; Alison et al., 2000 Lagasse et al., 2000). Although the interpretation of these experiments require re-examination in light of other recent experiments (Ying et al., 2002; Terada et al., 2002), it remains important to establish whether there exists a common stem cell for liver and pancreas, and whether there are pathways of transdifferentiation in addition to that established so far.

Although some of the details of the transdifferentiation of pancreas to liver have now been established, some questions still remain to be answered. In particular, the role of C/EBPβ (and other members of the C/EBP family) in normal liver development is poorly understood. For example, the expression patterns of C/EBPs shall be determined during embryonic development. It is also necessary to determine

whether hepatocytes can arise from other pancreatic cell types apart from exocrine cells and whether the transdifferentiation of exocrine cells to hepatocytes has any real significance to the situation in humans.

REFERENCES

1. Ahlgren, U., Jonsson, J., Jonsson, L., Simu, K., and Edlund, H. (1998). Beta-cell-specific inactivation of the mouse Ipf1/Pdx1 gene results in loss of the beta-cell phenotype and maturity onset diabetes. Genes Dev *12*, 1763-8.

2. Alison, M.R., Poulsom, R., Jeffery, R., Dhillon, A.P., Quaglia, A., Jacob, J., Novelli, M., Prentice, G., Williamson, J., Wright, N.A. (2000). Hepatocytes from non-hepatic adult stem cells. Nature *406*, 257.

3. Alison, M. (1998). Liver stem cells: a two compartment system. Curr. Opin. Cell Biol. *10*, 710-5.

4. Araki, M. and Okada, T.S. (1977). Differentiation of lens and pigment cells in cultures of neural retinal cells of early chick embryos. Dev Biol *60*, 278-86.

5. Arizmendi, C., Liu, S., Croniger, C., Poli, V., and Friedman, J.E. (1999). The transcription factor CCAAT/enhancer-binding protein beta regulates gluconeogenesis and phosphoenolpyruvate carboxykinase (GTP) gene transcription during diabetes. J Biol Chem *274*, 13033-40.

6. Almind, K., Inoue, G., Pedersen, O., and Kahn, C.R. (1996). A common amino acid polymorphism in insulin receptor substrate-1 causes impaired insulin signaling. Evidence from transfection studies. J. Clin. Invest. *97*, 2569-2575.

7. Barden, T.P. and Knowles, H.C. Jr (1981). Diagnosis of diabetes in pregnancy. Clin Obstet Gynecol *24*, 3-19.

8. Beck, F., Chawengsaksophak, K., Waring, P., Playford, R.J., and Furness, J.B. (1999). Reprogramming of intestinal differentiation and intercalary

regeneration in Cdx2 mutant mice. Proc Natl Acad Sci U S A *96*, 7318-23.

9. Bell, G.I., Pilkis, S.J., Weber, I.T., and Polonsky, K.S. (1996). Glucokinase mutations, insulin secretion, and diabetes mellitus. Annu. Rev. Physiol. *58*, 171-186

10. Benediktsson, R., Calder, A.A., Edwards, C.R., and Seckl, J.R. (1997). Placental 11 beta-hydroxysteroid dehydrogenase: a key regulator of fetal glucocorticoid exposure. Clin Endocrinol *46*, 161-6.

11. Benediktsson, R., Lindsay, R.S., Noble, J., Seckl, J.R., and Edwards, C.R. (1993). Glucocorticoid exposure in utero: new model for adult hypertension. Lancet *341*, 339-41.

12. Ben-Ze'ev, A., Robinson, G.S., Bucher, N.L.R., and Farmer, S.R., (1998). Cell-cell and cell-matrix interactions differently regulate the expression of hepatic and cytoskeletal genes in primary cultures of rat hepatocytes. Proc Natl Acd Sci USA *85*, 2161-2165.

13. Beresford, W.A. (1990). Direct transdifferentiation: can cells change their phenotype without dividing? Cell Differ Dev *29*, 81-93.

14. Billis, A. and Rastogi, G.K. (1966). Studies in methods of investigating carbohydrate. Diabetologia *2*, 169-77.

15. Birchmeier, C. and Gherardi, E. (1998). Developmental roles of HGF/SF and its receptor, the c-Met tyrosine kinase. Trends Cell Biol *8*, 404-10.

16. Blondeau, B., Lesage, J., Czernichow, P., Dupouy, J.P., and Bre'ant B. (2001). Glucocorticoids impair fetal b-cell development in rats. Am J Physiol

Endocrinol Metab 281, E592-E599.

17. Bossard, P., McPherson, C.E., and Zaret, K.S. (1997). In vivo footprinting with limiting amounts of embryo tissues: a role for C/EBP beta in early hepatic development. Methods *11*, 180-8.

18. Bossard, P. and Zaret, K.S. (2000). Repressive and restrictive mesodermal interactions with gut endoderm: possible relation to Meckel's Diverticulum. Development *127*, 4915-23.

19. Bouwens, L. (1998). Cytokeratins and cell differentiation in the pancreas. J Pathol *184*, 234-9.

20. Bouwens, L. (1998). Transdifferentiation versus stem cell hypothesis for the regeneration of islet beta-cells in the pancreas. Microsc Res Tech *43*, 332-6.

21. Boyko, E.J. (2000). Proportion of type 2 diabetes cases resulting from impaired fetal growth. Diabetes Care *23*, 1260-4.

22. Brembeck, F.H., Kaiser, A., Detjen, K., Hotz, H., Foitzik, T., Buhr, H.J., Riecken, E.O., and Rosewicz, S. (1998). Retinoic acid receptor alpha mediates growth inhibition by retinoids in rat pancreatic carcinoma DSL-6A/C1 cells. Br J Cancer *78*, 1288-95.

23. Byrne, C.D. and Phillips, D.I. (2000). Fetal origins of adult disease: epidemiology and mechanisms. J Clin Pathol *53*, 822-8.

24. Campbell A.L., and Murphy B.E.P. (1977). The maternal-fetal cortisol gradient during pregency and at delivery. J Clin Endocrinol Metab *45*, 435-440

25. Caplin, M.E., Clarke, P., Grimes, S., Dhillon, A.P., Khan, K., Savage, K., Lewin, J., Michaeli, D., Pounder, R.E., and Watson, S.A. (1999). Demonstration of new sites of expression of the CCK-B/gastrin receptor in pancreatic acinar AR42J cells using immunoelectron microscopy. Regul Pept *84*, 81-9.

26. Cascio, S. and Zaret, K.S. (1991). Hepatocyte differentiation initiates during endodermal-mesenchymal interactions prior to liver formation. Development *113*, 217-25.

27. Cereghini, S., Ott, M.O., Power, S., and Maury, M. (1992). Expression patterns of vHNF1 and HNF1 homeoprotein in early postimplantation embryos suggest distinct and sequential development roles. Development *116*, 783-797.

28. Cereghini, S. (1996). Liver-enriched transcription factors and hepatocyte differentiation. FASEB J *10*, 267-282.

29. Chen, W.S., Manova, K., Weinstein, D.C., Duncan, S.A., Plump, A.S., Prezioso, V.R., Bachvarova, R.F., and Darnell. J.E. (1994). Disruption of the HNF-4 gene, expressed in visceral endoderm, leads to cell death in embryonic ectoderm and impaired gastrulation of mouse embryos. Genes Dev *8*, 2466-2477.

30. Chou, J.Y., Wan, Y.-J.Y., and Sakiyama, T. (1988). Regulation of rat liver maturation in vitro by glucocorticoids. Mol. Cell Biol *8*, 203-209.

31. Christophe, J. (1994). Pancreatic tumoral cell line AR42J: an amphicrine model. Am J Physiol *266*, G963-G971.

32. Clark, P.M., Hindmarsh, P.C., Shiell, A.W., Law, C.M., Honour, J.W., and Barker, D.J. (1996). Size at birth and adrenocortical function in childhood.

Clin Endocrinol *45*, 721-6.

33. Corish, P., and Tyler-Smith, C. (1999). Attenuation of green fluorescent protein half-like in mammalian cells. Protein Eng *12*, 1035-1040

34. Dabeva, M.D., Hurston, E., and Sharitz, D.A. (1995). Transcription factor and liver-specific mRNA expression in facultative epithelial progenitor cells of liver and pancreas. Am J Pathol *147*, 1633-48.

35. Dabeva, M.D., Hwang, S.G., Vasa, S.R., Hurston, E., Novikoff, P.M., Hixson, D.C., Gupta, S., and Shafritz, D.A. (1997). Differentiation of pancreatic epithelial progenitor cells into hepatocytes following transplantation into rat liver. Proc Natl Acad Sci U S A *94*, 7356-61.

36. Darlington, G.J. (1999). Molecular mechanisms of liver development and differentiation. Curr Opin Cell Biol *11*, 678-82.

37. Darlington, G.J., Ross, S.E., and MacDougald, O.A. (1998). The Role of C/EBP Genes in Adipocyte Differentiation. J Biol Chem *273*, 30057-30060.

38. Debas, H.T. (1997). Molecular insights into the development of the pancreas. Am J Surg *174*, 227-31.

39. Defronzo, M., (1997). Pathogenesis of type II diabetes: metabolic and molecular implication for identifying diabetes genes. Diabetes Rev *5*, 177-269

40. Descombes, P., and Schibler, U.A (1991). liver-enriched and transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. Cell *67*, 569-579.

41. Delaunay, F., Khan, A., Cintra, A., Davani, B., Ling, Z.C., Andersson, A., Ostenson, C.G., Gustafsson, J., Efendic, S., and Okret, S. (1997). Pancreatic beta cells are important targets for the diabetogenic effects of glucocorticoids. J Clin Invest *100*, 2094-8.

42. Dethloff, L., Barr, B., Bestervelt, L., Bulera, S., Sigler, R., LaGattuta, M., and de La Iglesia, F. (2000). Gabapentin-induced mitogenic activity in rat pancreatic acinar cells. Toxicol Sci *55*, 52-9.

43. Deutsch, G., Jung, J., Zheng, M., Lora, J., and Zaret, K.S. (2001). A bipotential precursor population for pancreas and liver within the embryonic endoderm. Development *128*, 871-81.

44. Diehl, A.M., (1998). Role of CCAAT/Enhancer binding proteins in regulation of liver regenerative growth. J Biol Chem *273*, 30843-40846.

45. DiPersio, C.M., Jackson, D.A., and Zaret, K.S. (1991). The extracellular matrix coordinately modulates liver transcription factors and hepatocyte morphology. Mol Cell Biol *11*, 4405-14.

46. Duncan, S.A., Nagy, A., and Chan, W. (1997). Murine gastrulation requires HNF-4 regulated gene expression in the visceral endoderm: tetraploid rescue of Hnf-$4^{-/-}$ embryos. Development *124*, 279-287.

47. Duncan, S.A., Navas, M.A., Dufort, D., Rossant, J., and Stoffel, M. (1998). Regulation of a transcription factor network required for differentiation and metabolism. Science *281*, 692-695.

48. Dunn, J.C.Y., Tompkins, R.G., and Yarmush, M.L. (1992). Hepatocytes in

collagen sandwich: evidence for transcriptional and translational regulation. J Cell Biol 116, 1043-1053.

49. Duvillie, B., Cordonnier, N., Deltour, L., Dandoy-Dron, F., Itier, J.M., Monthioux, E., Jami, J., Joshi, R.L., and Bucchini, D. (1997). Phenotypic alterations in insulin-deficient mutant mice. Proc Natl Acad Sci U S A *94*, 5137-40.

50. Edlund, H. (1999). Pancreas: how to get there from the gut? Curr Opin Cell Biol *11*, 663-8.

51. Edlund, H. (2001). Developmental biology of the pancreas. Diabetes *50 Suppl 1*, S5-9.

52. Edwards, C.R., Benediktsson, R., Lindsay, R.S., and Seckl, J.R. (1996). 11 beta-Hydroxysteroid dehydrogenases: key enzymes in determining tissue-specific glucocorticoid effects. Steroids *61*, 263-9.

53. Eguchi, G. and Okada, T.S. (1973). Differentiation of lens tissue from the progeny of chick retinal pigment cells cultured in vitro: a demonstration of a switch of cell types in clonal cell culture. Proc Natl Acad Sci U S A *70*, 1495-9.

54. Eguchi, G. (1975). [Induction of transdifferentiation in cultured cells - differentiation of pigment epithelial cells into crystalline lens cells]. Horumon To Rinsho *23*, 771-7.

55. Eguchi, G. and Kodama, R. (1993). Transdifferentiation. Curr Opin Cell Biol *5*, 1023-8.

56. Emens, L.A., Landers, D.W., and Moss, L.G. (1992). Hepatocyte nuclear factor 1α is expressed in a hamster insulinoma line and transactivates the rat insulin I gene. Proc Natl Acad Sci USA 89, 300-7304.

57. Fernandez-Mejia, C., Medina-Martinez, O., Martinez-Perez, L., and Goodman, P.A. (1999). The human insulin gene contains multiple transcriptional elements that respond to glucocorticoids. Pancreas *18*, 336-41.

58. Flanagan, D.E., Moore, V.M., Godsland, I.F., Cockington, R.A., Robinson, J.S., and Phillips, D.I. (2000). Fetal growth and the physiological control of glucose tolerance in adults: a minimal model analysis. Am J Physiol Endocrinol Metab *278*, E700-6.

59. Fowden, A.L. (1993). Insulin deficiency: effects on fetal growth and development. J Paediatr Child Health *29*, 6-11.

60. Giorgino, F., Almahfouz, A., Goodyear, L.J., and Smith, R.J. (1993). Glucocorticoid regulation of insulin receptor and substrate IRS-1 tyrosine phosphorylation in rat skeletal muscle in vivo. J Clin Invest *91*, 2020-30.

61. Gittes, G.K. and Rutter, W.J. (1992). Onset of cell-specific gene expression in the developing mouse pancreas. Proc Natl Acad Sci 89, 1128–1132.

62. Goodell M.A. (2001) Stem cells: is there a future in plastic? Curr Opin Cell Biol *13,* 662-665

63. Gremlich, S., Roduit, R., and Thorens, B. (1997). Dexamethasone induces posttranslational degradation of GLUT2 and inhibition of insulin secretion in isolated pancreatic beta cells. Comparison with the effects of fatty acids. J Biol

Chem *272*, 3216-22.

64. Grompe, M., Lindstedt, S., al-Dhalimy, M., Kennaway, N.G., Papaconstantinou, J., Torres-Ramos, C.A., Ou, C.N., and Finegold, M. (1995). Pharmacological correction of neonatal lethal hepatic dysfunction in a murine model of hereditary tyrosinaemia type I. Nat Genet *10*, 453-60.

65. Habener, J.F., and Hussain, M. (2001). Molecular basis of Pancreas development and function. Kluwer Academic Publishers

66. Hales, C.N. (1993). The role of poor fetal and infant growth in non-insulin-dependent diabetes. Bull Mem Acad R Med Belg *148*, 281-5; discussion 285-8.

67. Harris, D.M., Flannigan, K.L., Go, V.L., and Wu, S.V. (1999). Regulation of cholecystokinin-mediated amylase secretion by leptin in rat pancreatic acinar tumor cell line AR42J. Pancreas *19*, 224-30.

68. Hattersley, A.T. and Tooke, J.E. (1999). The fetal insulin hypothesis: an alternative explanation of the association of low birthweight with diabetes and vascular disease. Lancet *353*, 1789-92.

69. Hayashi, Y., Wang, W., Ninomiya, T., Nagano, H., Ohta, K., and Itoh, H. (1999). Liver enriched transcription factors and differentiation of hepatocellular carcinoma. Mol Pathol *52*, 19-24.

70. Herrera, P.L., Huarte, J., Sanvito, F., Meda, P., Orci, L., and Vassalli, J.D. (1991). Embryogenesis of the murine endocrine pancreas: Early expression of pancreatic polypeptide gene. Development *113*, 1257–1265.

71. Hill, D.E. (1976). Insulin and fetal growth. Prog Clin Biol Res *10*, 127-39.

72. Hill, D.J., Petrik, J., and Arany, E. (1998). Growth factors and the regulation of fetal growth. Diabetes Care *21 Suppl 2*, B60-9.

73. Hill, D.J. and Duvillie, B. (2000). Pancreatic development and adult diabetes. Pediatr Res *48*, 269-74.

74. Holtzer, H., Rubinstein, S., Fellini, S., Yeoh, G., Chi, J., Birnbaum, J., and Okayawa, M. (1975). Lineages, quantal cell cycles, and the generation of cell diversity. Q Rev Biophys *8*, 523-557

75. Horb, L.D. and Slack, J.M. (2000). Role of cell division in branching morphogenesis and differentiation of the embryonic pancreas. Int J Dev Biol *44*, 791-6.

76. Horikawa, Y., Iwasaki, N., Hara, M., Furuta, H., Hinokio, Y., Cockburn, B., Lindner, T., Yamagata, K., Ogata, M., Tomonaga, O., Kuroki, H., Kasahara, T., Iwamoto, Y., and Bell, G.I. (1997). Mutation in hepatocyte nuclear factor-1 beta gene (TCF2) associated with MODY. Nat. Genet. *17*, 384-385

77. Hruban, R.H., Molina, J.M., Reddy, M.N., and Boitnott, J.K. (1987). A neoplasm with pancreatic and hepatocellular differentiation presenting with subcutaneous fat necrosis. Am J Clin Pathol *88*, 639-45.

78. Ide, H., Subbarao, V., Reddy, J.K., and Rao, M.S. (1993). Formation of ductular structures in vitro by rat pancreatic epithelial oval cells. Exp Cell Res *209*, 38-44.

79. Imai, Y., Philippe, N., Sesti, G., Accili, D., and Taylor, S.I. (1997). Expression

of variant forms of insulin receptor substrate-1 identified in patients with noninsulin-dependent diabetes mellitus. J. Clin. Endocrinol. Metab. *82*, 4201-4207.

80. Isom, H.C., Secott, T., Georgoff, I., Woodowrth, C., and Mummaw, J. (1985). Manitenance of differentiated rat hepatocytes in primary culture. Proc Natl Acad Sci USA *82*, 3252-3256.

81. Jeffers, M., Rao, M.S., Rulong, S., Reddy, J.K., Subbarao, V., Hudson, E., Vande Woude, G.F., and Resau, J.H. (1996). Hepatocyte growth factor/scatter factor-Met signaling induces proliferation, migration, and morphogenesis of pancreatic oval cells. Cell Growth Differ *7*, 1805-13.

82. Jefferson, D.M., Clayton, D.F., Darnell, J.E., Jr., and Reid, L.M. (1984). Post-transcriptional modulation of gene expression in cultured rat hepatocytes. Mol Cell Biol *4*, 1929-1934.

83. Jonsson, J., Carlsson, L., Edlund, T., and Edlund, H. (1994). Insulin-promoter-factor 1 is required for pancreas development in mice. Nature *371*, 606-9.

84. Jung, J., Zheng, M., Goldfarb, M., and Zaret, K.S. (1999). Initiation of mammalian liver development from endoderm by fibroblast growth factors. Science *284*, 1998-2003.

85. Kadowaki, T., Tamemoto, H., Tobe, K., Terauchi, Y., Ueki, K., Kaburagi, Y., Yamauchi, T., Satoh, S., Sekihara, H., Aizawa, S., and Yazaki, Y. (1996). Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1 and identification of insulin receptor substrate-2. Diabet Med *13*, S103-8.

86. Kahn, B.B. (1998). Type 2 diabetes: when insulin secretion fails to compensate for insulin resistance. Cell *92*, 593-6.

87. Kamiya, A., Kinoshita, T., Ito, Y., Matsui, T., Morikawa, Y., Senba, E., Nakashima, K., Taga, T., Yoshida, K., Kishimoto, T., and Miyajima, A. (1999). Fetal liver development requires a paracrine action of oncostatin M through the gp130 signal transducer. EMBO J *18* , 2127-36

88. Kaufman, M.H., and Barp, J.B.L (2000). The anatomical basis of mouse development. Academic Press.

89. Kerneis, S. and Pringault, E. (1999). Plasticity of the gastrointestinal epithelium: the M cell paradigm and opportunism of pathogenic microorganisms. Semin Immunol *11*, 205-15.

90. Kim, S.K. and Hebrok, M. (2001). Intercellular signals regulating pancreas development and function. Genes Dev *15*, 111-27.

91. Kinoshita, T., Sekiguchi, T., Xu, M.J., Ito, Y., Kamiya, A., Tsuji, K., Nakahata, T., and Miyajima, A. (1999). Hepatic differentiation induced by oncostatin M attenuates fetal liver hematopoiesis. Proc Natl Acad Sci U S A *96*, 7265-70.

92. Kodama, R. and Eguchi, G. (1995). From lens regeneration in the newt to in-vitro transdifferentiation of vertebrate pigmented epithelial cells. Semin Cell Biol *6*, 143-9.

93. Kojima, N., Kinoshita, T., Kamiya, A., Nakamura, K., Nakashima, K., Taga, T., and Miyajima, A. (2000). Cell density-dependent regulation of hepatic development by a gp130- independent pathway. Biochem Biophys Res

Commun *277*, 152-8.

94. Konishi, N., Ward, J.M., and Waalkes, M.P. (1990). Pancreatic hepatocytes in Fischer and Wistar rats induced by repeated injections of cadmium chloride. Toxicol Appl Pharmacol *104*, 149-56.

95. Krakowski, M.L., Kritzik, M.R., Jones, E.M., Krahl, T., Lee, J., Arnush, M., Gu, D., and Sarvetnick, N. (1999). Pancreatic expression of keratinocyte growth factor leads to differentiation of islet hepatocytes and proliferation of duct cells. Am J Pathol *154*, 683-91.

96. Kramer, B. and Penny, C. (2001). Regulation of embryonic chick insulin cells: effect of retinoic acid and insulin-like growth factor 1. Cells Tissues Organs *169*, 42-8.

97. Kulkarni, R.N., Bruning, J.C., Winnay, J.N., Postic, C., Magnuson, M.A., and Kahn, C.R. (1999). Tissue-specific knockout of the insulin receptor in pancreatic beta cells creates an insulin secretory defect similar to that in type 2 diabetes. Cell *96*, 329-39.

98. Kuo, C.J., Conley, P.B., Chen, L., Sladek, F.M., Darnell, J.E., and Crabtree, G.R. (1992). A transcriptional hierarchy involved in mammalian cell-type specification. Nature *355*, 457-461.

99. Lagasse, E., Connors, H., Al-Dhalimy, M., Reitsma, M., Dohse, M., Osborne, L., Wang, X., Finegold, M., Weissman, I.L., Grompe, M. (2000). Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. Nat Med *6*, 1229-34.

100. Lambillotte, C., Gilon, P., and Henquin, J.C. (1997). Direct glucocorticoid

inhibition of insulin secretion. An in vitro study of dexamethasone effects in mouse islets. J Clin Invest *99* , 414-23.

101. Langley-Evans, S.C., Phillips, G.J., Benediktsson, R., Gardner, D.S., Edwards, C.R., Jackson, A.A., and Seckl, J.R. (1996). Protein intake in pregnancy, placental glucocorticoid metabolism and the programming of hypertension in the rat. Placenta *17*, 169-72.

102. Lee, B.C., Hendricks, J.D., and Bailey, G.S. (1989). Metaplastic pancreatic cells in liver tumors induced by diethylnitrosamine. Exp Mol Pathol *50*, 104-13.

103. Levitt, N.S., Lindsay, R.S., Holmes, M.C., and Seckl, J.R. (1996). Dexamethasone in the last week of pregnancy attenuates hippocampal glucocorticoid receptor gene expression and elevates blood pressure in the adult offspring in the rat. Neuroendocrinology *64*, 412-8.

104. Li, J., Ning, G., and Duncan, S.A. (2000). Mammalian hepatocyte differentiation requires the transcription factor HNF4α. Genes Dev *14*, 464-474.

105. Lindsay, R.S., Lindsay, R.M., Edwards, C.R., and Seckl, J.R. (1996). Inhibition of 11-beta-hydroxysteroid dehydrogenase in pregnant rats and the programming of blood pressure in the offspring. Hypertension *27*, 1200-4.

106. Lindsay, R.S., Lindsay, R.M., Waddell, B.J., and Seckl, J.R. (1996). Prenatal glucocorticoid exposure leads to offspring hyperglycaemia in the rat: studies with the 11 beta-hydroxysteroid dehydrogenase inhibitor carbenoxolone. Diabetologia *39*, 1299-305.

107. Liu, J.K., DiPersio, C.M., and Zaret, K.S. (1991). Extracellular signals that regulate liver transcription factors during hepatic differentiation in vitro. Mol Cell Biol *11*, 773-84.

108. Liu, S., et al. (1998). Targeted disruption of C/EBP beta gene results in hypoglycemia and impaired glucagon-stimulated glucose production. Diabetes *47*, A295-A295 Suppl.

109. Logsdon, C.D., Moessner, J., William, J.A., and Goldfine, I.D. (1985). Glucocorticoids increase amylase mRNA levels, secretory organelles, and secretion in pancreatic acinar AR42J cells. J Cell Biol *100*, 1200-1208

110. Longnecker, D.S., Lilja, H.S., French, J.I., Kuhlmann, E., and Noll. W. 1979. Transplantation of azaserine-induced carcinomas of pancreas in rats. Cancer Lett *7*, 197-202.

111. Lu, M., Seufert, J., and Habener J.F. (1998). Pancreatic b-cell-specific repression of insulin gene transcription by CCAAT/Enhancer-binding protein b. J. Biol. Chem. 272, 28349-28359.

112. Makino, T., Usuda, N., Rao, S., Reddy, J.K., and Scarpelli, D.G. (1990). Transdifferentiation of ductular cells into hepatocytes in regenerating hamster pancreas. Lab Invest *62*, 552-61.

113. Mashima, H., Ohnishi, H., Wakabayashi, K., Mine, T., Miyagawa, J.-I., Hanafusa, T., Sena, M., Yamada, H., Kojima, I. (1996a). Betacellulin and Activin A corrdinately convert amylase-secreting pancreatic AR42J cells into insulin-secreting cells. J Clin Invest *97*, 1647-1654.

114. Mashima, H., Shibata, H., Mine, T., and Kojima, I. (1996b). Formation of insulin-producing cells from pancreatic acinar AR42J cells by hepatocyte growth factor. Endocrinology *137*, 3969-3976.

115. Matsukura, N., Suzuki, K., Kaeachi, T., Aoyagi, M., Sugimura, T., Kitaoka, H., Numajiri, H., Shirota, A., Habashi, M., and Hirota, T. (1980).Distribution of maker enzymes and mucin in intestinal metaplasia in human stomach and relation of complete and incomplete metaplasia to minute gastric carcinomas. J. Natl Cancer inst *65*, 231-40

116. McDevitt, D.S. (1989). Transdifferentiation in animals. A model for differentiation control. Dev Biol *6*, 149-73.

117. Michael M. D., Kulkarni, R.N., Postic, C., Previs, S.T., Shulman, G.I., Magnuson, M.A., and Kahn, C.R. (2000). Loss of insulin signalling in hepatocytes leasds to servere insulin resistance and progressive hepatic dysfunction. Cell *6,* 87-97.

118. Michalopoulos, G.K., and DeFrances M.C. (1997). Liver regeneration. Science *276*, 60-66.

119. Miquerol L., Lopez, S., Cartier, N., Tulliez, M., Raymondjean, M., and Kahn. A. (1994). Expression of the L-type pyruvate kinase gene and the hepatocyte nuclear factor 4 transcription factor in exocrine and endocrine pancreas. J Biol Chem 269, 8944-8951.

120. Milner, R.D. and Hill, D.J. (1984). Fetal growth control: the role of insulin and related peptides. Clin Endocrinol *21*, 415-33.

121. Milos, P.M. and Zaret, K.S. (1992). A ubiquitous factor is required for C/EBP-related proteins to form stable transcription complexes on an albumin promoter segment in vitro. Genes Dev *6*, 991-1004.

122. Monis, B., Valentich, M.A., Urrutia, R., and Rivolta. M. (1991). Multicentric focal acinar cell hyperplasia and hepatocyte-like cell metaplasia are induced by nitrosomethylurea in rat pancreas. Int J Pancreatol *8*, 119-131.

123. Moriya, N., Komazaki, S., Takahashi, S., Yokota, C., and Asashima, M. (2000). In vitro pancreas formation from Xenopus ectoderm treated with activin and retinoic acid. Dev Growth Differ *42*, 593-602.

124. Nagy, P., Bisgaard, H.C., and Thorgeirsson, S.S. (1994). Expression of hepatic transcription factors during liver development and oval cell differentiation. J Cell Biol *126*, 223-233.

125. Nomura, K., Takagi, S., and Okada, T.S. (1980). Expression of neuronal specificities in "transdifferentiating" cultures of neural retina. Differentiation *16*, 141-7.

126. Nyirenda, M.J., Lindsay, R.S., Kenyon, C.J., Burchell, A., and Seckl, J.R. (1998). Glucocorticoid exposure in late gestation permanently programs rat hepatic phosphoenolpyruvate carboxykinase and glucocorticoid receptor expression and causes glucose intolerance in adult offspring. J Clin Invest *101*, 2174-81.

127. Nyirenda, M.J. and Seckl, J.R. (1998). Intrauterine events and the programming of adulthood disease: The role of fetal glucocorticoid exposure (Review). Int J Mol Med *2*, 607-614.

128. Okada, T.S., Yasuda, K., Araki, M., and Eguchi, G. (1979). Possible demonstration of multipotential nature of embryonic neural retina by clonal cell culture. Dev Biol *68*, 600-17.

129. Okada, T.S. (1980). Cellular metaplasia or transdifferentiation as a model for retinal cell differentiation. Curr Top Dev Biol *16*, 349-80.

130. Okada, T.S. (1983). Recent progress in studies of the transdifferentiation of eye tissue in vitro. Cell Differ *13*, 177-83.

131. Okada, T.S. Transdifferentiation. Flexibility in Cell Differentiation. Oxford; Clarendon Press (1991).

132. Paner, G.P., Thompson, K.S., and Reyes, C.V. (2000). Hepatoid carcinoma of the pancreas. Cancer *88*, 1582-9.

133. Penny, C. and Kramer, B. (2000). The effect of retinoic acid on the proportion of insulin cells in the developing chick pancreas. In Vitro Cell Dev Biol Anim *36*, 14-8.

134. Percival, A.C. and Slack, J.M. (1999). Analysis of pancreatic development using a cell lineage label. Exp Cell Res *247*, 123-32.

135. Petersen, B.E., Bowen, W.C., Patrene, K.D., Mars, W.M., Sullivan, A.K., Murase, N., Boggs, S.S., Greenberger, J.S., and Goff, J.P. (1999). Bone marrow as a potential source of hepatic oval cells. Science *284*, 1168-70.

136. Phillips, D.I., Barker, D.J., Hales, C.N., Hirst, S., and Osmond, C. (1994a). Thinness at birth and insulin resistance in adult life. Diabetologia *37*, 150-4.

137. Phillips, D.I., Hirst, S., Clark, P.M., Hales, C.N., and Osmond, C. (1994b). Fetal growth and insulin secretion in adult life. Diabetologia *37*, 592-6.

138. Phillips, D.I. (1998). Birth weight and the future development of diabetes. A review of the evidence. Diabetes Care *21 Suppl 2*, B150-5.

139. Pour, P.M. and Schmied, B.M. (1999). One thousand faces of Langerhans islets. Int J Pancreatol *25*, 181-93.

140. Ramiya, V.K., Maraist, M., Arfors K.E., Schatz, D.A., Peck, A.B., and Cornelius, J.G. (2000). Reversal of insulin-dependent diabetes using islets generated *in virto* from pancreatic stem cells. Nat Med *6*, 278-282.

141. Rao, M.S., Reddy, M.K., Reddy, J.K., and Scarpelli, D.G. (1982). Response of chemically induced hepatocytelike cells in hamster pancreas to methyl clofenapate, a peroxisome proliferator. J Cell Biol *95*, 50-6.

142. Rao, M.S., Subbarao, V., Luetteke, N., and Scarpelli, D.G. (1983). Further characterization of carcinogen-induced hepatocytelike cells in hamster pancreas. Am J Pathol *110*, 89-94.

143. Rao, M.S., Bendayan, M., Kimbrough, R.D., and Reddy, J.K. (1986a). Characterization of pancreatic-type tissue in the liver of rat induced by polychlorinated biphenyls. J Histochem Cytochem *34*, 197-201.

144. Rao, M.S., Scarpelli, D.G., and Reddy, J.K. (1986b). Transdifferentiated hepatocytes in rat pancreas. Curr Top Dev Biol *20*, 63-78.

145. Rao, M.S., Subbarao, V., and Reddy, J.K. (1986c). Induction of hepatocytes in the pancreas of copper-depleted rats following copper repletion. Cell Differ

18, 109-17.

146. Rao, M.S., Subbarao, V., Yeldandi, A.V., and Reddy, J.K. (1987). Pancreatic acinar cell regeneration following copper deficiency-induced pancreatic necrosis. Int J Pancreatol *2*, 71-85.

147. Rao, M.S., Dwivedi, R.S., Subbarao, V., Usman, M.I., Scarpelli, D.G., Nemali, M.R., Yeldandi, A., Thangada, S., Kumar, S., and Reddy, J.K. (1988a). Almost total conversion of pancreas to liver in the adult rat: a reliable model to study transdifferentiation. Biochem Biophys Res Commun *156*, 131-6.

148. Rao, M.S., Subbarao, V., and Scarpelli, D.G. (1988b). Development of hepatocytes in the pancreas of hamsters treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin. J Toxicol Environ Health *25* , 201-5.

149. Rao, M.S., Dwivedi, R.S., Yeldandi, A.V., Subbarao, V., Tan, X.D., Usman, M.I., Thangada, S., Nemali, M.R., Kumar, S., Scarpelli, D.G., et al (1989). Role of periductal and ductular epithelial cells of the adult rat pancreas in pancreatic hepatocyte lineage. A change in the differentiation commitment. Am J Pathol *134*, 1069-86.

150. Rao, M.S., Yeldandi, A.V., and Reddy, J.K. (1990). Differentiation and cell proliferation patterns in rat exocrine pancreas: role of type I and type II injury. Pathobiology *58*, 37-43.

151. Rao, M.S., Subbarao, V., Sato, K., and Reddy, J.K. (1991). Alterations of pancreatic hepatocytes in rats exposed to carcinogens. Am J Pathol *139*, 1111-7.

152. Rao, M.S. and Reddy, J.K. (1995). Hepatic transdifferentiation in the pancreas. Semin Cell Biol *6*, 151-6.

153. Rao, M.S., Yeldandi, A.V., and Reddy, J.K. (1990). Stem cell potential of ductular and periductular cells in the adult rat pancreas. Cell Differ Dev *29*, 155-63.

154. Rao, M.S., Yeldandi, A.V., Subbarao, V., and Reddy, J.K. (1993). Role of apoptosis in copper deficiency-induced pancreatic involution in the rat. Am J Pathol *142*, 1952-7.

155. Rao, M.S., Yukawa, M., Omori, M., Thorgeirsson, S.S., and Reddy, J.K. (1996). Expression of transcription factors and stem cell factor precedes hepatocyte differentiation in rat pancreas. Gene Expr *6*, 15-22.

156. Reddy, J.K., Kanwar, Y.S., Rao, M.S., Watanabe, T.K., Reddy, M.K., Parsa, I., Longnecker, D.S., and Tafuri, S. (1986). Duct-like morphogenesis of Longnecker pancreatic acinar carcinoma cells maintained in vitro on seminiferous tubular basement membranes. Cancer Res *46*, 347-54.

157. Reddy, J.K., Rao, M.S., Qureshi, S.A., Reddy, M.K., Scarpelli, D.G., and Lalwani, N.D. (1984). Induction and origin of hepatocytes in rat pancreas. J Cell Biol *98*, 2082-90.

158. Reddy, J.K., Rao, M.S., Yeldandi, A.V., Tan, X.D., and Dwivedi, R.S. (1991). Pancreatic hepatocytes. An in vivo model for cell lineage in pancreas of adult rat. Dig Dis Sci *36*, 502-9.

159. Reddy, M.K., Heda, G.D., and Reddy, J.K. (1987). Purification and

characterization of alpha-amylase from rat pancreatic acinar carcinoma. Comparison with pancreatic alpha-amylase. Biochem J *242*, 681-7.

160. Reinisch J.M., Simon, N. G., Karwo, W.G., and Gandleman R. (1978). Prenatal exposure to prednisone in humans and animals retards intra-uterine growth. Science *202*, 436-438.

161. Richards, C.D., Brown, T.J., Shoyab, M., Baumann, H. and Gauldie, J. (1992). Recombinant oncostatin M stimulates the production of acute phase proteins in HepG2 cells and rat primary hepatocytes *in vitro*. J Immunol *148*, 1731–1736.

162. Roebuck, B.D., Baumgartner, K.J., Thron, C.D., and Longnecker, D.S. (1984). Inhibition by retinoids of the growth of azaserine-induced foci in the rat pancreas. J Natl Cancer Inst *73*, 233-6.

163. Rørth, P., and Montell, D.J. (1992). Drosophila C/EBP: a tissue-specific DNA-binding protein required for embryonic development. Gene Dev *6*, 2299-2311.

164. Rosenberg, L. (1995). In vivo cell transformation: neogenesis of beta cells from pancreatic ductal cells. Cell Transplant *4*, 371-83.

165. Rose,T. M., and Bruce, A.G. (1991). Oncostatin M is a member of a cytokine family that includes leukemia-inhibitory factor, granulocyte colony-stimulating factor, and interleukin 6. Proc Natl Acad Sci *USA*, *88*, 8641–8645.

166. Rutter, G.A. (2000). Diabetes: the importance of the liver. Curr Biol *10*, R736-8.

167. Samadani, U., and Costa, R. (1996). The transcription activator hepatocyte nuclear factor 6 regulates liver gene expression. Mol Cell Biol 16, 6273-84.

168. Scarpelli, D.G. and Rao, M.S. (1981). Differentiation of regenerating pancreatic cells into hepatocyte-like cells. Proc Natl Acad Sci U S A *78*, 2577-81.

169. Scarpelli, D.G., Rao, M.S., and Reddy, J.K. (1991). Are acinar cells involved in the pathogenesis of ductal adenocarcinoma of the pancreas? Cancer Cells *3*, 275-7.

170. Screpanti, I., et al. (1995). Lymphoproliferative disorder and imbalanced T-helper response in C/EBP-beta-deficient mice. EMBO J 14, 1932-41.

171. Scharfmann, R. (2000). Control of early development of the pancreas in rodents and humans: implications of signals from the mesenchyme. Diabetologia *43*, 1083-92.

172. Schmidt, C., Bladt, F., Goedecke, S., Brinkmann, V., Zschiesche, W., Sharp, M., Gherardi, E., and Birchmeier, C (1995). Scatter factor/hepatocyte growth factor is essential for liver development. Nature *373*, 699-702.

173. Schmoll, D., Allan, B.B., and Burchell, A. (1996). Cloning and sequencing of 5' region of the human glucose-6-phosphatase gene: transcriptional regulation by camp, insulin and glucocorticoid in H4IIE hepatoma cells. FEBS Lett *383*, 63-66

174. Seckl, J.R. (1997). Glucocorticoids, feto-placental 11 beta-hydroxysteroid dehydrogenase type 2, and the early life origins of adult disease. Steroids *62*,

89-94.

175. Seckl, J.R., Cleasby, M., and Nyirenda, M.J. (2000). Glucocorticoids, 11beta-hydroxysteroid dehydrogenase, and fetal programming. Kidney Int *57*, 1412-7.

176. Seckl, J.R., Nyirenda, M.J., Walker, B.R., and Chapman, K.E. (1999). Glucocorticoids and fetal programming. Biochem Soc Trans *27*, 74-8.

177. Sell S (2001). Heterogeneity and plasticity of hepatocyte lineage cells. Hepatology 33, 738-50.

178. Selman, K. and Kafatos, F.C. (1974). Transdifferentiation in the labial gland of silk moths: is DNA required for cellular metamorphosis? Cell Differ *3*, 81-94.

179. Seufert, J., Weir, G.C., and Habener, J.F. (1998). Differential expression of the insulin gene transcriptional repressor CCAAT/enhancer-binding protein beta and transactivator islet duodenum homeobox-1 in rat pancreatic beta cells during the development of diabetes mellitus. J Clin Invest *101*, 2528-39.

180. Sharma, S., Jhala, U.S., Johnson, T., Ferreri, K., Leonard, J., and Montminy, M. (1997). Hormonal regulation of an islet-specific enhancer in the pancreatic homeobox gene STF-1. Mol Cell Biol *17*, 2598-604.

181. Shen, C.N., Slack, J.M.W., and Tosh, D. (2000). Molecular basis of transdifferentiation of pancreas to liver. Nat Cell Biol *2*, 879-87.

182. Simone, V.D., and Cortese, R. (1992). Transcription factors and liver-specific genes. Biochimica Biophysica Acta *1132*, 119-126.

183. Sirica, A.E. (1995). Ductular hepatocytes. Histol Histopathol *10*, 433-56.

184. Slack, J.M.W. (1985). Homoeotic transformations in man: implications for the mechanism of embryonic development and for the organization of epithelia. J Theor Biol *114*, 463-90.

185. Slack, J.M.W. (1986). Epithelial metaplasia and the second anatomy. Lancet *2*, 268-71.

186. Slack, J.M.W. (1995). Developmental biology of the pancreas. Development *121*, 1569-80.

187. Slack, J.M.W. (2000). Stem cells in epithelial tissues. Science *287*, 1431-3.

188. Slack, J.M.W (2001). Essential developmental biology. Blackwell science.

189. Slack, J.M.W., and Tosh, D. (2001). Transdifferentiation and metaplasia-switching cell types. Curr Opin Genet Dev *11*, 581-586.

190. Sladek, F.M., Zhong, W.M., Lai, E., and Darnell. J.J. (1990). Liver-enriched transcription factor HNF-4 is a novel member of the steroid hormone receptor superfamily. Genes Dev *4*, 2353-2365.

191. Spath, G. F., & Weiss, M.C. Hepatocyte nuclear factor 4 expression overcomes repression of the hepatic phenotype in dedifferentiated hepatoma cells. *Mol. Cell. Biol.* 17, 1913-1922 (1997).

192. Spath, G. F., & Weiss, M. C. Hepatocyte nuclear factor 4 provokes expression of epithelial marker genes, acting as a morphogen in dedifferentiated hepatoma cells. *J. Cell Biol.* 140, 935-946 (1998).

193. Steiner, D.F., Tager, H.S., Chan, S.J., Nanjo, K., Sanke, T., and Rubenstein, A.H. (1990). Lessons learned from molecular biology of insulin-gene mutations. Diabetes care *13*, 600-609.

194. Stamatoglou, S.C. and Hughes, R.C. (1994). Cell adhesion molecules in liver function and pattern formation. FASEB J *8*, 420-7.

195. Stoffers, D., Ferrer, J., Clarke, W., and Habener, J. (1997). Early-onset type-II diabetes mellitus (MODY4) linked to IPF1. Nat. Genet. *17*, 138-139.

196. St-Onge, L., Wehr, R., and Gruss, P. (1999). Pancreas development and diabetes. Curr Opin Genet Dev *9*, 295-300.

197. Swift, G.H., Kruse, F., MacDonald, R.J., and Hammer, R.E. (1989). Differential requirements for cell-specific elastase I enhancer domains in transfected cells and transgenic mice. Genes Dev. *3*, 687-96.

198. Taga, T., and Kishimoto, T. (1997). Gp130 and the interleukin-6 family of cytokines. Annu Rev Immunol *15*, 797-819.

199. Tamemoto, H., Kadowaki, T., Tobe, K., Yagi, T., Sakura, H., Hayakawa, T., Terauchi, Y., Ueki, K., Kaburagi, Y., Satoh, S., and et, a.l. (1994). Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. Nature *372*, 182-6.

200. Tanaka, T., et al. (1997). Defective adipocyte differentiation in mice lacking the C/EBPbeta and/or C/EBPdelta gene. EMBO J. *16*, 7432-43.

201. Taub, R., Greenbaum, L.E., and Peng, Y. (1999). Transcriptional regulatory signals define cytokine-dependent and - independent pathways in liver

regeneration. Semin Liver Dis *19*, 117-27.

202. Taylor, D.J., Thompson, C.H., Kemp, G.J., Barnes, P.R., Sanderson, A.L., Radda, G.K., and Phillips, D.I. (1995). A relationship between impaired fetal growth and reduced muscle glycolysis revealed by 31P magnetic resonance spectroscopy. Diabetologia *38*, 1205-12.

203. Taylor, S.I. (1992). Lilly Lecture: molecular mechanisms of insulin resistance. Lessons from patients with mutations in the insulin-receptor gene. Diabetes *42*, 1473-1490.

204. Taylor, S.I. (1999). Deconstructing type 2 diabetes. Cell *97*, 9-12.

205. Terada, N., Hamazaki, T., Oka, M., Hoki, M., Mastalerz, D.M., Nakano, Y., Meyer, E.M., Morel, L., Petersen, B.E., and Scott, E.W. (2002). Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature *416,* 542-545.

206. Terauchi, Y., Tsuji, Y., Satoh, S., Minoura, H., Murakami, K., Okuno, A., Inukai, K., Asano, T., Kaburagi, Y., Ueki, K., et al (1999). Increased insulin sensitivity and hypoglycaemia in mice lacking the p85 alpha subunit of phosphoinositide 3-kinase. Nat. Genet. *21*, 230-236

207. Terauchi, Y., Kubota, N., Tamemoto, H., Sakura, H., Nagai, R., Akanuma, Y., Kimura, S., and Kadowaki, T. (2000). Insulin effect during embryogenesis determines fetal growth: a possible molecular link between birth weight and susceptibility to type 2 diabetes. Diabetes *49*, 82-6.

208. Thommesen, L., Norsett, K., Sandvik, A.K., Hofsli, E., and Laegreid, A.

(2000). Regulation of inducible cAMP early repressor expression by gastrin and cholecystokinin in the pancreatic cell line AR42J. J Biol Chem *275*, 4244-50.

209. Thompson, C.H., Sanderson, A.L., Sandeman, D., Stein, C., Borthwick, A., Radda, G.K., and Phillips, D.I. (1997). Fetal growth and insulin resistance in adult life: role of skeletal muscle morphology. Clin Sci *92*, 291-6.

210. Tosh, D., Shen, C.N., Alison, M.R., Sarraf, C.E., and J.M.W. Slack. (2002). Copper deprivation in rats induces islet hyperplasia and hepatic metaplasia in the pancreas *(In revision).*

211. Tronche, F., and Yaniv, M. (1992). HNF1, a homeoprotein member of the hepatic transcription regulatory network. Bioessays *14*, 579-587.

212. Tisch, R. and McDevitt, H. (1996). Insulin-dependent diabetes mellitus. Cell *85*, 291-7.

213. Tsanadis, G., Kotoulas, O., and Lollis, D. (1995). Hepatocyte-like cells in the pancreatic islets: study of the human foetal pancreas and experimental models. Histol Histopathol *10*, 1-10.

214. Usuda, N., Reddy, J.K., Hashimoto, T., and Rao, M.S. (1988). Immunocytochemical localization of liver-specific proteins in pancreatic hepatocytes of rat. Eur J Cell Biol *46*, 299-306.

215. Vasioukhin, V., Bauer, C., Yin, M., and Fuchs, E. (2000). Directed actin polymerization ds the driving force for epithelial cell–cell adhesion. Cell *100*, 209-219.

216. Waalkes, M.P., Cherian, M.G., Ward, J.M., and Goyer, R.A. (1992). Immunohistochemical evidence of high concentrations of metallothionein in pancreatic hepatocytes induced by cadmium in rats. Toxicol Pathol *20*, 323-6.

217. Wang, H., Maechler, P., Hagenfeldt, K.A., and Wollheim, C.B. (1998). Dominant-negative suppression of HNF-1α function results in defective insulin gene transcription and impaired metabolism-secretion coupling in a pancreatic β-cell line. EMBO J 17, 6701-6713.

218. Wang N.D., et al. (1995). Impaired Energy Homeostasis in C/EBP-alpha knockout mice. Science *269*, 1108-1112.

219. Wang, X., Al-Dhalimy, M., Lagasse, E., Finegold, M., and Grompe, M. (2001). Liver repopulation and correction of metabolic liver disease by transplanted adult mouse pancreatic cells. Am J Pathol *158*, 571-9.

220. Ward A.M. (1971). Glandular neoplasia within the urinary tract. The aetiology of adenocarcinoma of the urothelium with a review of the literature. Virchows Arch Pathol Anat *352*, 296-311.

221. White, M.F., and Yenush, L. (1998). The IRS-signaling system: a network of docking proteins that mediate insulin and cytokine action. Curr. Top. Microbiol. Immuno. *228*, 179-208

222. Wills, J., Watson, J.M., Hales, C.N., and Phillips, D.I. (1996). The relation of fetal growth to insulin secretion in young men. Diabet Med *13*, 773-4.

223. Wolf, H.K., Burchette, J.L. Jr, Garcia, J.A., and Michalopoulos, G. (1990).

Exocrine pancreatic tissue in human liver: a metaplastic process? Am J Surg Pathol *14*, 590-5.

224. Wolfe-Coote, S., Louw, J., Woodroof, C., and Du Toit, D.F. (1996). The non-human primate endocrine pancreas: development, regeneration potential and metaplasia. Cell Biol Int *20*, 95-101.

225. Yasuda, H., Tanaka, S., Ohnishi, H., Mashima, H., Ogushi, N., Mine, T., and Kojima, I. (1994). Activin A: negative regulator of amylase secretion and cell proliferation in rat pancreatic acinar AR42J cells. Am J Physiol *267*, G220-6.

226. Yamagata, K., N. Oda, P.J., Kaisaki, S., Menzel, H., Furuta, M., Vaxillaire, L., Southam, D., Cox, G.M., Lathrop, V.V., Boriraj, X., Chen, N.J., Cox, Y., Oda, H., Yano, M.M., Le Beau, S., Yamada, H., Nishigori, J., Takeda, S.S., Fajans, A.T., Hattersley, N., Iwasaki, T., Hansen, O., Pedersen, K.S., Polonsky, R.C., Turner, G., Velho, J.-C., Chevre, P., Froguel, K., and Bell, G.I. (1996a). Mutation in the hepatocyte nuclear factor-1□ gene in maturity-onset diabetes of the young (MODY3*)*. Nature *384*, 455-458.

227. Yamagata, K., H. Furuta, N. Oda, P.J. Kaisaki, S. Menzel, N.J. Cox, S.S. Fajans, S. Signorini, M. Stoffel, P., and Bell, G.I. (1996b). Mutation in the hepatocyte nuclear factor-4α gene in maturity-onset diabetes of the young (MODY1*)*. Nature *384*, 458-460.

228. Yeldandi, A.V., Milano, M., Subbarao, V., Reddy, J.K., and Rao, M.S. (1989). Evaluation of liver cell proliferation during ciprofibrate-induced hepatocarcinogenesis. Cancer Lett *47*, 21-7.

229. Yeldandi, A.V., Tan, X.D., Dwivedi, R.S., Subbarao, V., Smith, D.D. Jr,

Scarpelli, D.G., Rao, M.S., and Reddy, J.K. (1990). Coexpression of glutamine synthetase and carbamoylphosphate synthase I genes in pancreatic hepatocytes of rat. Proc Natl Acad Sci U S A *87*, 881-5.

230. Ying, Q.L., Nichols, J., Evens, E.P., and Smith, A.G. (2002). Changing potency by spontaneous fusion. Nature *416*, 545-548.

231. Zaret, K.S. (1996). Molecular genetics of early liver development. Annu Rev Physiol *58*, 231-51.

232. Zaret, K.S. (1998). Early liver differentiation: genetic potentiation and multilevel growth control. Curr Opin Genet Dev *8*, 526-31.

233. Zaret, K.S. (2000). Liver specification and early morphogenesis. Mech Dev *92*, 83-8.